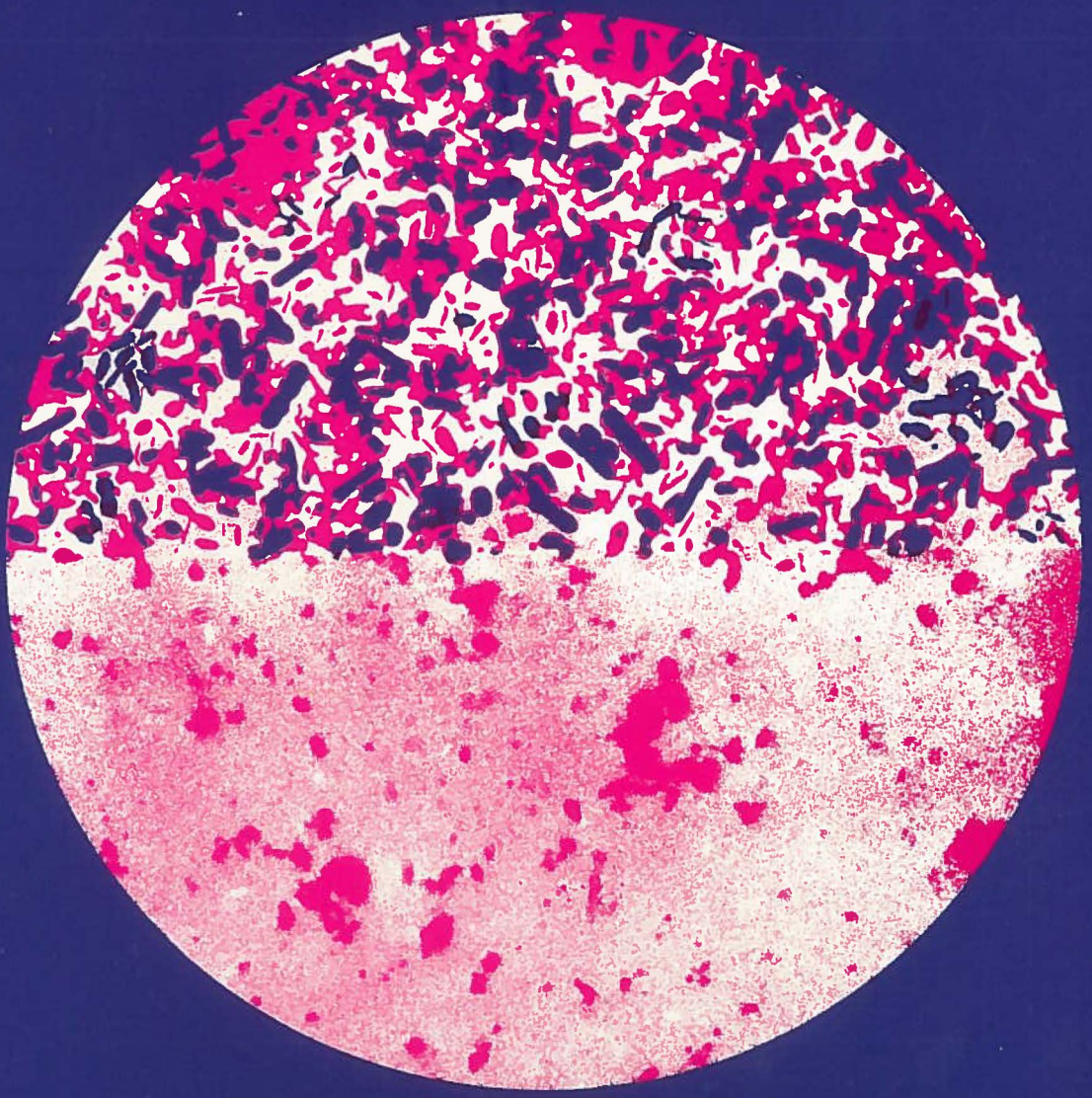
GASTROINTESTINAL DECONTAMINATION IN HEALTHY AND LETHALLY IRRADIATED MONKEYS



W. D. H. Hendriks

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STELLINGEN

I

Gastrointestinale decontaminatie met niet-resorbeerbare antibiotica leidt niet tot een kiemvrije status.

II

Gastrointestinale decontaminatie mag niet zonder strikte omgekeerde isolatie worden uitgevoerd.

III

Bij gastrointestinale decontaminatie moet na het staken van de niet-resorbeerbare antibiotica de kolonisatie resistentie worden hersteld door de darm met een geschikte anaerobe microflora te koloniseren alvorens de isolatie kan worden beëindigd.

IV

Strikte omgekeerde isolatie bij patiënten lijdende aan irreversibele immunodeficiënties is ethisch alleen verantwoord indien er een passende donor voor beenmergtransplantatie beschikbaar is.

V

Bij selectieve decontaminatie verdient het aanbeveling kiemarm voedsel te verstrekken.

VI

Beenmergtransplantaties tussen donor/ontvanger combinaties die verschillen voor antigenen van het hoofd-histocompatibiliteits complex leiden niet tot een lethale graft-versus-host reactie indien het transplantaat is ontdaan van immunocompetente lymfocyten en de ontvanger succesvol is gedecontamineerd.

VII

Bij rapportage over de effectiviteit van antimicrobiële prophylaxe dient te worden vermeld dat de conclusie alleen geldig is voor de onderzochte populatie gedurende de tijd dat het onderzoek werd verricht.

VIII

Bij ingrepen waarbij het colon wordt geopend zijn ter preventie van wondinfecties naast een mechanische reiniging van de darm parenteraal toegediende antibiotica even effectief als orale darmflorasuppressie, mits tijdens de ingreep reeds adequate weefselspiegels aanwezig zijn.

IX

Uit het feit dat bij virologisch onderzoek van patiënten, in het bijzonder die met virale meningitis, uiterst zelden poliovirus wordt geïsoleerd mag niet worden geconcludeerd dat dit virus in de bevolking niet circuleert.

X

De nog steeds hoge morbiditeit ten gevolge van de z.g. "andere salmonellosen" wijst erop, dat de aanbevelingen uit de rapporten van de Gezondheidsraad ter zake uit 1962 en 1978 niet adequaat tot uitvoering (kunnen) worden gebracht.

XI

Thrombocyten en granulocyten spelen een belangrijke rol in de klaring van endotoxine uit het bloed.

XII

Een chromosomale analyse van lymfoïde cellen bij patiënten die een orgaantransplantatie zullen ondergaan zou inzicht kunnen verschaffen in de kans op ontwikkeling van een lymfoïde maligniteit bij deze patiënten.

XIII

Het gebruik van geprogrammeerde systemen (zoals b.v. API) voor moeilijk te determineren stammen leidt tot verlies aan inzicht in de systematiek en tot verminderd plezier in het werk.

Groningen, 28 mei 1980
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Willem Dirk Huibertus Hendriks

geboren te Aalsmeer

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Aan mijn moeder

Aan Joke
Suzanne
Bart

CONTENTS

CHAPTER I

INTRODUCTION

In patients suffering from inborn immune defects (Koning et al., 1969; Bortin and Rimm, 1977; Kenny and Hitzig, 1979), aplastic anaemia (Storb et al., 1974; 1978; Bone Marrow Transplant Registry, 1976) and (terminal) leukaemia (UCLA Bone Marrow Transpl. Team, 1977; Thomas et al., 1977; Bortin and Rimm, 1978), bone marrow transplantation can be a life-saving treatment, although infections appear to be among the limiting factors (Van Bekkum and De Vries, 1967; Clift et al., 1974; Winston et al., 1979).

The occurrence of infections in individuals undergoing bone marrow transplantation is the result of a diminished function of the immune system which is caused not only by the basic disease but also by the pretreatment for the bone marrow transplantation. This pretreatment, which can consist of total body irradiation, chemotherapy or a combination of these, is necessary for the suppression of the patient's own immune system. The immunosuppression is required because otherwise the grafted bone marrow will be rejected (Host versus Graft reaction). After the grafting has been performed, it takes time before the haemopoietic tissues become repopulated with the grafted cells and some restoration of the immune functions becomes evident (Rådl et al., 1974; Storb et al., 1976). It is obvious that infections can easily develop in the interval in which the phagocytic and immune functions are minimal.

When the graft becomes immunologically active against the host, this results in a so-called Graft versus Host disease. The normal epithelial lining of skin and intestine is damaged, while other organs such as the liver can also be affected (Van Bekkum and De Vries, 1967). The defective epithelial lining between the body and the outside (microbial) world facilitates the development of infections in a situation where the immune functions are even more depressed (Noel et al., 1978).

If an infection occurs, it should be treated by administration of appropriate bactericidal antibiotics. However, clinical experience has shown that chances for succesful therapy and survival of the patients are poor unless granulocytes reappear in the circulation and retain

their normal function. If not, granulocyte transfusions as available in some centers nowadays may be beneficial. However, these should be obtained from appropriate donors and should be given for a sufficient time interval (Schiffer, 1977). It is obvious that this situation calls for measures to prevent the development of infections.

Infections can be due to microorganisms that were acquired during hospitalization (exogenous infections) or to microorganisms already part of the patient's own microflora at admission (endogenous infections). Exogenous infections can be reduced by protective isolation. The patient is separated from the normal environment in an attempt to prevent microorganisms with which there is no previous immunological experience from colonizing him (Bagshaw, 1964). The prevention of infection from exogenous sources leaves the endogenous microflora unaffected. This endogenous microflora can also become invasive, particularly when the normal epithelial lining is damaged in these immunologically compromised individuals as mentioned above.

The events leading to endogenous infections in immunodeficient individuals were studied in animal studies. It was found in conventional mice, that, after a total body irradiation, microorganisms could frequently be isolated from the mesenteric lymph nodes several days before they appeared in the blood (Van der Waaij et al., 1972a; 1978). These microorganisms belonged to the normal (endogenous) intestinal flora of the mice. Administration of antibiotics for eradication of these invading microorganisms have the side effect that the antibiotics also affect the normal flora (Van der Waaij, 1972b; Thijm and Van der Waaij, 1979). This suggests that systemic antibiotic treatment greatly facilitates the colonization by microorganisms resistant to the antibiotics applied. Complete elimination of the endogenous microflora should prevent these infections.

In mice, elimination of the microflora can be accomplished by adding nonabsorbable antibiotics to the drinking water (Van der Waaij et al., 1971a). When this treatment is successful, the mice have much in common with germfree mice (Van der Waaij, 1969; Gustafsson and Norin, 1977), the enlarged caecum being the most impressive similar feature.

Germfree mice are known to be very sensitive to colonization by exogenous microorganisms. Any microorganism introduced into their isolator will colonize the animals. It will grow out into an abnormally high concentration in the faeces and can even become invasive. There is a similar sensitivity to microbial colonization in decontaminated mice (Van der Waaij et al., 1971b); therefore, decontamination must be

carried out under circumstances of strict reverse isolation as developed in germfree research.

Isolation methods suitable for germfree research were established by Gustafsson (1948) and Reyniers (1959). They developed steam sterilizable stainless steel isolators connected to a small autoclave that functioned as an entry lock for introduction of materials into the isolators. Maintaining germfree animals became much easier and less expensive when plastic isolators became available (Trexler, 1959). This type of isolator is used in many modifications nowadays for reverse isolation of human patients (Schwartz and Perry, 1966; Dietrich et al., 1969; Trexler et al., 1975; Wilson and Mastromarino, 1977). These patient isolators consist of a plastic enclosure fitted with air inlets and outlets, sluices for introduction and removal of materials and are provided with gloves or half-suites for manipulation inside the isolator. This design has major disadvantages: a) any microbe that leaks into the isolator may settle anywhere inside; b) they consist of one chamber, prohibiting resterilization of the interior for as long as the individual is inside; and c) the interior is rather inaccessible for technical procedures.

Another type of isolator, which does not have these disadvantages, is based on the principle generally known as "laminar air flow". The latter, if not disturbed by large items, prevents introduction of microorganisms from the outside into the isolator. All microbes disseminated inside the isolator and suspended in the air are rapidly evacuated. Dressed in sterile surgical gowns, personnel have easier and more direct access to the isolated individuals. No transfer of microorganisms to these individuals will occur as long as the attendants remain downstream of the isolated individual and apply sterile techniques. The efficacy of laminar air flow cabinets for the maintenance of germfree mice has been described (Van der Waaij and Andreas, 1971c) and, since these mice could be maintained germfree for an undefined period in these isolators, a two-chamber downflow isolator was developed for isolation of patients. The advantage of the two-chamber system is that the patient can enter while the "conventional flora" is still present. Oral antibiotic supply will eliminate the latter from the patient but will leave the interior of the isolator contaminated (by contact contamination). One chamber can be sterilized while the patient is in the other compartment (Van der Waaij et al., 1973a).

In germfree animals, it was found that the intestinal microflora in mammals as well as in birds was dispensable (Luckey, 1963a). For these germfree animals, however, the food had to be enriched with proteins and vitamins in order to overcome the losses due to the ste-

rilization procedure and the altered intestinal physiology. When these supplements are provided, mice and rats thrive and breed well (Luckey, 1963a).

When germfree mice were conventionalized by placing them in a conventional animal house, there was often a significant loss of animals due to infection (Luckey, 1963c). In the complete absence of the microflora, virtually every living microorganism that is ingested will colonize the animal intestines in an abnormal pattern, as mentioned above. In conventional animals, the resident microflora protects against colonization of the intestinal tract. This phenomenon, which is called Colonization Resistance (C.R.) is attributed to the anaerobic part of the microflora (Van der Waaij, 1971b).

When gastrointestinal decontamination by nonabsorbable antibiotics can be discontinued because the immune functions are restored to a sufficient extent, the antibiotics are discontinued. Before such an animal or patient is taken out of isolation (reconventionalized), it is of advantage to purposely introduce an anaerobic microflora that provides a good colonization resistance. This will enable the individual to withstand a (low dose) contamination with potentially pathogenic microorganisms in the normal (conventional) environment.

In our institute, mice are kept "clean-conventional" (Hollander, 1976). Their aerobic microflora is relatively simple in composition and no antibiotics are added to the food, so that there is no selection for resistant strains. The absence of more resistant microorganisms facilitates the elimination of the gastrointestinal microflora. The differences in anatomy and physiology of the intestinal tract of rodents as well as the above-mentioned difference in sensitivity of the microflora prohibits a direct extrapolation of data found in experiments with these rodents to the human (patient) situation.

The development of a model closer to man was needed not only to investigate the prevention of endogenous and exogenous infections in such animals but also to further study the observation that transplantations of incompatible allogeneic bone marrow in germfree and decontaminated mice do take without necessarily causing clinical Graft versus Host disease (Jones et al., 1971; Van Bekkum et al., 1974b; Heit et al., 1977). Also if spleen cells were added to make the graft more comparable to primate bone marrow in these mice experiments, it delayed the appearance of fatal Graft versus Host disease (Van Bekkum, 1974b), even when the mice were recontaminated and reconventionalized (Van Bekkum, 1977b).

In this work the aspects of gnotobiotic treatment will be discussed. The three main subjects are: 1) how to eliminate the microflora and what are the side effects; 2) how to maintain the gnotobiotic state even after total body irradiation and what are the side effects in this treatment interval; 3) how to discontinue the decontamination and restore the "normal" microflora allowing reconventionalization.

In Chapter II, the efficacy in the elimination of the gastrointestinal microflora in healthy monkeys will be discussed. After elimination of the microflora, a monkey should remain free of microorganisms; therefore, the isolation efficacy will also be discussed. Both the efficacy in elimination of microorganisms and the isolation efficacy determine the possibilities for obtaining a monkey "free of microorganisms".

The agents applied for decontamination are potentially toxic. In elimination of the normal microflora, alterations in the intestinal physiology are induced. Side effects on liver, kidney and bone marrow function were therefore monitored. Since all monkeys developed diarrhoea, electrolyte levels were also followed. These aspects will be discussed in Chapter III.

After irradiation, the microflora should remain suppressed. The efficacy in maintaining the monkeys free of colonizations by microorganisms will be discussed in Chapter IV. Obviously, sterile cultures are not continuously obtained from all monkeys. It is therefore of importance to have some knowledge of the clinical relevance of a positive sample. This will be discussed in Chapter V. The diarrhoea increases after irradiation. The problems encountered in maintaining electrolyte levels will be discussed in Chapter VI.

When the gastrointestinal microflora is to be suppressed, the antibiotic concentration should be well above the minimum inhibitory concentration. Faeces is the only material available for study on a regular basis. The faecal antibiotic concentration was determined and the results are described in Chapter VII.

When the immune system had been restored to such extent that colonizations of the gastrointestinal tract could be dealt with, the antibiotics were discontinued and a nonpathogenic microflora was administered in order to restore the colonization resistance (Chapter VIII). Because of previous infections, systemic antibiotics sometimes have to be administered. In a mouse model the effects of several antibiotics on the donor flora were studied (Chapter IX).

As mentioned above, the work was done with rhesus monkeys in order to develop a model for bone marrow transplantation as close to humans as possible. Working with monkeys, however, is expensive and the animals are available in limited numbers. The monkeys were therefore incorporated into other studies simultaneously. This resulted in several subgroups leading to rather inhomogenous material. The group of monkeys described will be indicated in each chapter. For the reasons mentioned above, no conventional controls were included in order to compare the number of infections.

CHAPTER II

GASTROINTESTINAL DECONTAMINATION AND REVERSE ISOLATION IN HEALTHY MONKEYS

2.1 INTRODUCTION

In intensive myelotoxic chemotherapy for malignancies or in total body irradiation as a preparation for bone marrow grafting, the risk of infection is often a limiting factor. The invading microorganisms usually belong to the group of facultative anaerobic Gram-negative rods which colonize the oro-gastrointestinal tract in these patients. The microorganisms involved are frequently acquired from the hospital environment (Schimpff et al., 1972). Systemic antibiotic treatment often modifies the normal flora and thereby facilitates invasion and colonization by microorganisms resistant to the antibiotics applied (Van der Waaij et al., 1972b).

In an attempt to overcome these limitations, a number of approaches varying from relatively "simple" reverse isolation procedures (Bagshaw, 1964) to more strict isolation in laminar air flow systems have been explored (Lidwell and Towers, 1972; Van der Waaij et al., 1973a; and others). Protection against colonization and invasion by exogenous potentially pathogenic microorganisms does not decrease the risk of septic complications caused by microorganisms from the patients endogenous flora. For this reason, the patients are treated with oral nonabsorbable antibiotics in several centres in the world in an attempt to eliminate the endogenous gastrointestinal microflora (Bodey et al., 1968a; Levi et al., 1973; Levine et al., 1973; Yates and Holland, 1973; Trexler et al., 1975; Schimpff et al., 1975; Dietrich et al., 1977). In many of these studies, difficulties have been encountered in obtaining sterile cultures from the oropharynx of the treated patients and particularly in the elimination of yeasts from the oropharynx and the stools. Similar problems were encountered in bone marrow transplantation in rhesus monkeys. It was therefore dedecided to study these aspects in the monkeys.

2.2 MATERIALS AND METHODS

2.2.1 Monkeys

Fifty-three Macaca mulatta (rhesus) monkeys weighing between 1800 and 3000 grams were obtained from the colony of the Primate Center TNO. The animals were free of endogenous pathogens such as Salmonella spp., Shigella spp. and Mycobacterium tuberculosis (Van Bekkum and Van der Waaij, 1971). They were weighed daily and a blood sample was taken twice a week for determination of haematocrit, leukocyte reticulocyte, and thrombocyte count as well as transaminases* (Chapter III).

2.2.2 Isolation procedure

Each monkeys was housed in a separate cage in a double-chamber laminar air flow (L.F.) cabinet. For handling and during the cleaning of the cages, the monkeys were placed in a restraint chair inside the reserve chamber (Fig.2.1). After the cage and the chamber were cleaned with a disinfecting soap, they were sterilized by spraying with a 2% solution of peracetic acid (P.A.A.). This cleaning and sterilization procedure was performed daily. During the study the procedure was slightly modified; the cages were no longer cleaned inside the cabinets but were replaced every other day by clean P.A.A. sterilized ones. These were sterilized with P.A.A. outside the L.F. cabinet in a special fogging cabinet.

All items to be introduced into the L.F. isolator were paper-wrapped and steam sterilized. Packaged goods were opened with aseptic precautions in front of the L.F. cabinet. Bottles with sterile contents were sprayed externally with P.A.A. immediately before introduction.

During handling inside the L.F. cabinet, the technician wore a face mask, long neoprene gloves and a plastic apron. The gloves and apron were sterilized by spraying with P.A.A. immediately before handling inside the L.F. and between the handling of different animals. In order to also include recent colonizations in the sensitivity test, isolation was effected at least 24 h before the first faecal samples were taken.

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* This study was done in cooperation with the Dept. of Pediatrics of Leiden University Hospital, where all biochemical tests were performed (Dr. M.A.H. Giesberts).

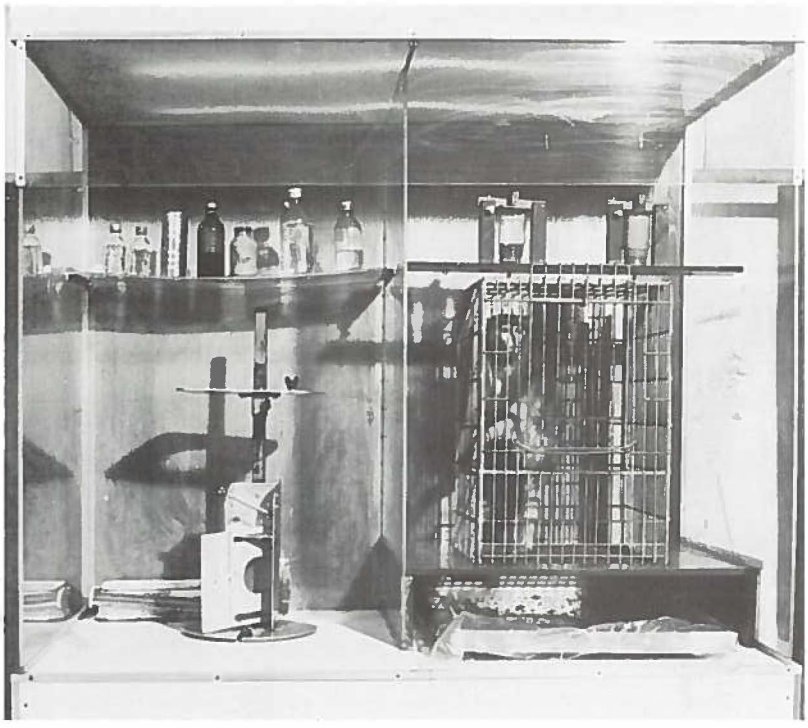

Figure 2.1:

The laminar air flow isolator

2.2.3 Food

To assure a constant antibiotic intake without taking the monkeys out of their cages and placing them in a restraint chair to force them to swallow the preparations, the antibiotics were mixed with a liquid diet (see Antibiotic regimen). The monkeys drank this mixture from 180 ml drinking bottles provided with 5 cm long nipples and which were placed on top of the cages. The bottles and nipples were cleaned and sterilized daily. The formula of the diet is given in Table 2.I. The intake was 2 ± 1 bottle per day. When an animal did not drink this diet spontaneously (as often occurred in the first days of antibiotic treatment), it was fed by stomach tube. A catether designed for umbilical vein catetherisation in neonates was passed through the nostrils into the stomach. After verification of the correct position of the tip by introduction of a few ml of saline, the food was slowly introduced into the stomach. In this way, the monkeys received ± 60 ml

Table 2.I

COMPOSITION OF THE LIQUID DIET*

	Fat	Protein	Carbohydrate	Minerals	J.100 ml^{-1}
Nutrison[1] 40 ml	4 %	4 %	11.3%	1 %	420
Farilacid[2] 40 ml	2.7%	5.9%	19.3%	1.3%	579.6
Carvam Cevitam[3] 20 ml	-	-	57.8%	1 %	1234.8
Glucose 10% 40 ml	-	-	10 %	-	176.4
KCl 10% 10 ml	-	-	-	10 %	-
Water to 180 ml	-	-	-	-	-
Total mixture	1.5%	2.2%	15.4%	1.2%	398.6

* as indicated by the manufacturers
1 Nutrison: a complete stomach feeding containing spore elements and vitamins (Nutricia, Holland)
2 Farilacid: an acidified baby milk formula (Nutricia, Holland)
3 Carvan Cevitam: a baby fruit syrup (Zwaardemaker, Holland)

of the liquid diet 3-4 times a day. A volume of 2-5 ml of the mixture was then also left in the oral cavity in order to decontaminate the oropharyngeal area.

2.2.4 Drinking water

Tap water was obtained from the "hot water supply". To limit microbial growth, hydrochloric acid was added to adjust the pH to 3. After standing overnight, the water was put into the drinking trough, which was also cleaned and sterilized daily.

2.2.5 Selection of the most suitable antibiotic combination

According to our conclusion in a previous publication (Hendriks et al., 1975), only the first part of the sensitivity test as described by Van der Waaij et al. (1970) was performed. A cephalosporin antibiotic* was tested (on 3 occasions, it was replaced by bacitracin) with neomycin, dihydrostreptomycin, kanamycin and, on some occasions, gentamicin or tobramycin* in a checkerboard titration: 0.5 ml of Brain Heart Infusion broth (B.H.I.) (Oxoid) to which 100 IU of nystatin (La Baz) per ml was added was pipetted into a plastic tray of 64 cups of 2 ml volume. A checkerboard titration was then performed in squares of 16 cups. The antibiotic concentrations as indicated in Fig. 2.2 were obtained by adding 0.05 ml of a stock solution containing 250 mg of the antibiotic per ml into the first row of cups and by diluting it serially for three steps with diluting loops of 0.05 ml capacity (Flow Laboratories). The other antibiotic of each combination was diluted in a similar way in a perpendicular direction. One drop of a 1:10 suspension of faeces was then added to each cup by means of a Pasteur pipet. After overnight incubation at 37° C, subinoculation was performed with a stamp onto Endo agar (Difco) for culturing Gram-negative bacteria, onto aesculin-azide agar for culturing Streptococcus faecalis and onto B.H.I. agar (Difco) for nonselective culturing. These plates were subsequently incubated overnight at 37° C and observed for growth. That combination of antibiotics which was bactericidal in the highest number of cups was selected for treatment. When all antibiotic combina-

* Cephalosporin antibiotics were kindly supplied by Glaxo BV (Cepalorin®) and Eli Lilly Inc. (Keflin®, Keflodin®), which also made the tobramycin (Obracin®) available.

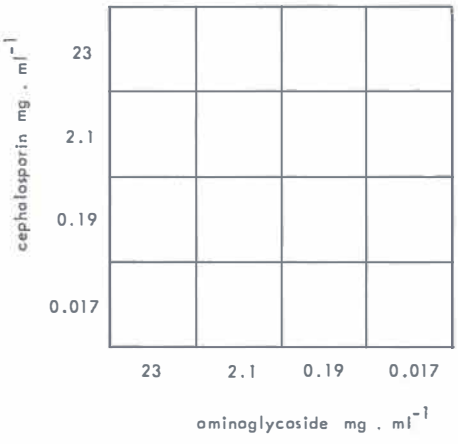

Figure 2.2:

Scheme of a tray with "checkerboard titration" of antibiotics.

Each square represents one cup of 2 ml; one tray consists of 4 of these blocks. Gentamicin and tobramycin were only available in vials of 40 mg.ml^{-1}. This was used in combination with stock solution of 250 mg.ml^{-1} cephalosporin.

tions were bactericidal in all cups, the choice was based on availability of the antibiotics.

2.2.6 Antibiotic regimen

The presence of the normal anaerobic microflora exhibiting the colonization resistance capacity facilitates the suppression of yeasts by antifungal agents; therefore, to more effectively eliminate yeasts from the gastrointestinal tract, 600,000 IU of nystatin was added daily to the liquid food beginning at least three days prior to the start of oral antibacterial treatment.

The antimicrobial treatment consisted of the oral administration of 0.5 gram of each of the selected antibiotics along with 600,000 IU of nystatin three times daily for the first three days and twice daily for two more days (the high dose treatment period) (Table 2.II). Metronidazole (Flagyl^{R}) was given in a dose of 125 mg twice a day in order to eliminate intestinal flagellates. It might also have affected the anaerobic bacteria in the intestines (Chow et al., 1975; Willis et al., 1976).

The selected antibiotics and nystatin were also added to the liquid diet in amounts of 1 or 2.5 mg, respectively 120,000 - 300,000 IU, per ml from the first day of antibacterial treatment onwards to ensure a rather constant dose. In 28 monkeys, the cephalosporin was re-

Table 2.II

ORAL ANTIBIOTIC ADMINISTRATION FOR GASTROINTESTINAL DECONTAMINATION

300,000 IU nystatin.ml^{-1} at least 3 days in liquid food in otherwise not yet treated animals
500 mg of antibiotic A+B and nystatin (600,000 IU) 3 times/day for 3 days
500 mg of antibiotic A+B and nystatin (600,000 IU) 2 times/day for 2 days
from the first day of antibiotic administration 1 - 2.5 mg of antibiotic A + B and 120,000-300,000 IU nystatin.ml^{-1} in liquid food.

A: aminoglycoside
B: cephalosporin or bacitracin

placed by bacitracin between days 9 and 22 for economic reasons. In 6 animals, bacitracin instead of a cephalosporin was added to the food from the first day of treatment onwards.

When colonization with a particular microorganism occurred, the antibiotics were adjusted to the sensitivity pattern of that microorganism as determined by the standard disk diffusion test.

2.2.7 Microbiological monitoring

Beginning before the first antibiotic administration, swab samples were taken daily from the oral cavity, the ears, the hands, the chest and the perianal area. The cotton swabs were premoistened in B.H.I. broth. After sampling, they were incubated at 37^{o} C in the same broth tube as used for moistening. A loopful of fresh faeces (about 10 mg) was inoculated into 9 ml of Brewer's thioglycolate medium (Difco) for incubation. In this way, the existing antibiotic concentration was diluted by a factor of about 1000, i.e., to below the minimum inhibitory concentration (M.I.C.) of microorganisms in the sample in many cases (Chapter VII). The culture tubes were scored as negative when no growth was observed after at least 3 days of incubation at 37^{o} C. Although living bacteria may have been present by that time, they were considered as unimportant, in terms of both causing infections and controlling the intestinal flora.

In the presentation of the data, the sampled sites (ear, hand and chest) were pooled and recorded as "skin". The perianal swab was included along with the faecal cultures.

During the first two years of this study, a Gram-stained faecal smear was made daily for microscopic observation in order to detect the presence of microorganisms as early as possible. However, after it was found that living microorganisms were generally isolated from the oral cavity before the Gram-stain of the faeces became positive, as indicated by the presence of microorganisms, the Gram-staining was replaced by daily semiquantitative culturing of the oropharynx. For this purpose, a swab sample of the oral cavity was streaked onto a segment of a B.H.I.agar plate; with the inoculation needle, subsequent segments were streaked. After overnight aerobic incubation at 37° C, growth only in the first segment was scored as 1^+, in only the first and second segments as 2^+, etc.

When growth occurred in one of the broth cultures of the samples, subinoculations onto appropriate agar media were made for identification and for sensitivity testing. The antibiotic regimen was then readjusted according to the results of the agar diffusion sensitivity test. When a Candida species was isolated, in some monkeys, amphotericin B* instead of nystatin was administered orally in a daily dose of 50-100 $mg^{-1}.monkey^{-1}.day^{-1}$.

A monkey was considered to be colonized with a particular microorganism when the same species was isolated at least twice from either of the sampling sites within seven days. Microorganisms isolated at day 7 of treatment were considered to result from incomplete decontamination. Microorganisms isolated for the first time after this period were attributed to a "break in the isolation procedure".

2.3 RESULTS

The antibiotic treatment was very well tolerated. The mean weight gain of 10 monkeys that were treated for more than 40 days was almost 200 grams, which is within the normal range. In none of the monkeys was an increase in urea or S.G.P.T. which could be attributed to the antibiotics observed (Chapter III).

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* Amphotericin B (Fungizone®) was kindly supplied by Squibb.

Table 2.III

ANTIBIOTIC COMBINATIONS USED FOR DECONTAMINATION

Combination	Number of monkeys
Neomycin - Cephalosporin	38
Dihydrostreptomycin - Cephalosporin	12
Neomycin - Bacitracin	1
Tobramycin - Bacitracin	2
Total	53

2.3.1 Efficacy of decontamination

The antibiotic combination to be administered always inhibited growth in a concentration of 0.19 $mg.ml^{-1}$ or less in the sensitivity test. The combinations employed are presented in Table 2.III.

As soon as the antibiotics could be detected in the faeces, a mild diarrhoea (soft faeces) developed and the microflora of all sampled sites was greatly reduced in number as well as in variety of species.

The interval between the initiation of oral antibiotic treatment and the day at which the faecal cultures and swabs (including the one of the oral cavity) were sterile for at least 3 consecutive days is presented in Table 2.IV. After discontinuation of the high dose regimen in 6 monkeys, microorganisms appeared in the cultures of the samples. These could be suppressed.

Table 2.IV

DURATION OF ANTIBIOTIC TREATMENT PRECEDING STERILE CULTURES

Treatment period in days	Number of monkeys	Per cent of total number of monkeys
1	13	24.5
2	7	13.2
3	12	22.6
4	2	3.8
No sterile cultures obtained at day 7	19	35.9
Total	53	100

Table 2.V

COLONIZATIONS THAT COULD BE SUPPRESSED AFTER UNSUCCESSFUL DECONTAMINATION
(in 11 out of 53 monkeys)

First isolation on treatment day	Microorganism		
	Gram-positive	Gram-negative	Yeast
1	-	2	1
2	2	2	-
3	-	-	-
4	1	-	-
5	1	2	-
6	-	3	-

Gram-positive: 1 Staph. aureus, 3 Strept. faecalis.
Gram-negative: 7 Enterobacteriaceae, 2 non-fermenting rods (excl. Pseudomonas). 3 monkeys were colonized by 2 microorganisms.

The microorganisms that were still present at day 7 of treatment, i.e., on the third day after termination of the "high dose treatment". are presented in Tables 2.V and 2.VI. In Table 2.V, those cases in which the microorganism could be eliminated by therapeutic interference are indicated. Those cases are presented in which this was not possible despite adjustments made in the antibiotic treatment are presented in Table 2.VI.

In 3 monkeys in which decontamination was started with a combination of an aminoglycoside with bacitracin instead of a cephalosporin antibiotic, cultures never became sterile. Of 6 animals in which treatment was started with combinations including bacitracin in the liquid food and a cephalosporin antibiotic in the high dose period, the microflora was completely suppressed in only one monkey.

Table 2.VI

COLONIZATIONS PERSISTENT FROM THE FIRST WEEK OF TREATMENT ONWARDS
(in 16 out of 53 monkeys)

First isolation on treatment day	Microorganism		
	Gram-positive	Gram-negative	Yeast
1	-	4	3
2	1	1	1
3	-	1	3
4	-	-	2

Gram-negative: non-fermenting rods (excl. Pseudomonas), with one exception susceptible to the aminoglycoside. All colonizations involved the oral cavity, two non-fermenting rods were initially also isolated from other sites.

2.3.2 Breaks in isolation

The 53 monkeys were under antibiotic treatment in the isolation facilities for a total of 1620 days (range 15-72; median 27 days). If all isolations of microorganisms after day 7 of treatment of a monkey are considered as a "break in isolation", we experienced a total of 45 breaks in isolation involving 33 monkeys. These are presented in Table 2.VII. Their distribution in time is presented in Table 2.VIII.

In the great majority of the breaks, the microorganism was first isolated from the oral cavity and, in a few monkeys, also from the perianal region and/or the faeces, but rarely was the initial isolation made from the skin swabs only. When microorganisms were isolated from the oral cavity, they were found to be present in limited numbers, as indicated by the semiquantitative culturing, except when the microorganism was resistant to the antibiotics applied. In these cases, all other sites were also found to be colonized.

Nineteen colonizations due to "breaks in isolation" did not result in a permanent colonization of the monkey (Table 2.IX). These "breaks" resulted in a few subsequent positive cultures. In 9 colonizations due to "breaks in isolation", adjustment of antibiotic treatment according to the sensitivity pattern of the microorganism resulted in suppression of the colonization.

The aim of decontamination was to achieve a complete suppression of the microflora in order to prevent microbial infections after lethal whole body irradiation. The results on obtaining such monkeys by decontamination under L.F. isolation conditions are presented in Table 2.X. In this Table, the number of colonizations at the end of the treatment period considered in this chapter are shown. As will be seen in Chapter IV, a number of minor colonizations that disappeared even after irradiation without adjustment of therapy are also included. The cultures growing *Pseudomonas* species became positive after irradiation; had they been known at the time of irradiation, the irradiation obviously would have been postponed.

2.4 DISCUSSION

Suppression of the microflora with an antibiotic combination selected by the sensitivity test was initially successful in over 60% of the animals within 4 days of treatment, i.e., in 34 out of 53 monkeys. However, in 6 of the 34 monkeys, microorganisms were isolated after discontinuation of the high dose regimen at day 4. In all 6 animals,

Table 2.VII

BREAKS IN ISOLATION
(expressed as number of monkeys colonized)

Microorganism:	Treatment period in days 8-15	15-22	22-29	29-36	⩾ 36	Total	Persistent colonization
Staphylococci	2	1	2	1	-	6	2
Streptococci (excl. Strept. faecalis)	2	1	-	-	-	3	3
Other Gram-positive microorganisms	4	2	2	-	2	10	2
Enterobacteriaceae	5	2	-	-	1	8	-
Pseudomonas	-	-	1	-	-	1	1
Other non-fermenting Gram-negative rods	-	2	1	-	1	4	1
Yeast	7	5	1	-	-	13	8
Total	20	13	7	1	4	45	17

Table 2.VIII

NUMBER OF MONKEYS, EXPOSURE TIME AND BREAKS IN ISOLATION

	Treatment period in days						
	0-8	8-15	15-22	22-29	29-36	⩾ 36	Total
Number of monkeys at beginning of period	53	53	51	33	22	17	
Number of days	371	371	292	203	128	255	1620
Number of breaks in isolation		20	13	7	1	4	45
Chance for a break in isolation per day		0.054	0.045	0.034	0.007	0.015	0.027

Table 2.IX

FATE OF COLONIZATIONS DUE TO A "BREAK IN ISOLATION"
(expressed as number of monkeys colonized)

	Total	Treatment period in days 8-15	15-22	22-29	29-36	> 36
Number of colonizations	45	20	13	7	1	4
Number that: disappeared without adjustment of treatment	19	3	7	4	1	4
disappeared after adjustment of treatment	9	9	-	-	-	-
could not be suppressed	17	8	6	3	-	-

the colonization could be controlled later. Microorganisms that persisted from the start of decontamination were most difficult to eradicate, particularly the yeasts and non-fermenting gram-negative rods. Therefore, the chance of success in suppression of the oro-gastro-intestinal microflora could be predicted after 4 days of treatment: isolation of non-fermenting rods or yeasts indicated a poor chance for complete suppression of the microflora. These microbes appeared to be of low pathogenicity. When kept at a low concentration, the yeasts did not become invasive (Chapter IV). One had to decide in each case whether treatment could be continued.

When not all sampled sites became sterile, the oral cavity frequently remained colonized. This could be explained by the many niches

Table 2.X

EFFICACY OF DECONTAMINATION

Colonizations present at irradiation or discontinuation of antibiotics:
(in 27 out of 53 monkeys)

Staphylococci	2
Streptococci (excl. Strept.faecalis)	3
Other Gram.positive microorganisms	3
Pseudomonas	1
Non-fermenting Gram-negative rods	7
Yeast	17
Total	33*

* 6 monkeys were colonized by 2 microorganisms

in the oropharynx, such as minor dental lesions, where microorganisms are shielded from the antimicrobial agents. Besides this, the exposure time of the bacteria to antibiotics was very short in this part of the digestive tract, i.e., the local antimicrobial concentration was well above the M.I.C. of the microorganisms for only a short period of time.

Our previous observation (Hendriks et al., 1975) that bacitracin should not be used in the initial phase of decontamination is confirmed by the present data. We had to apply this agent in three monkeys because cephalosporin antibiotic was not available at that time.

A number of failures of decontamination occurred in clusters, i.e., the non-fermenting Gram-negative rods, Serratia and yeasts were isolated from monkeys that were lodged simultaneously in the laminar air flow cabinets. We assume therefore that cross-contamination occurred in these cases. It is uncertain whether this took place before or during isolation, since these monkeys were obtained from the same animal room.

From this study, it is difficult to conclude whether a microorganism was present due to failure to eliminate it by antibiotic decontamination or due to a "break in isolation", since no typing of strains isolated before and during treatment could be made. Therefore, the assumption was made that the microorganisms appearing at days 6 and 7 were of endogenous origin and had escaped suppression due to a reduction in antibiotic intake. All microorganisms appearing after day 7 were considered to be the result of "breaks in the isolation". However, in a number of cases, persisting microorganisms may have become resistant to the antibiotics. It is known that yeasts can become stepwise gradually resistant to nystatin, as described in vitro (Athar and Winner, 1971) as well as in vivo in a bone marrow transplantation patient (Merz and Sandford, 1979). This may explain the relatively high number of "breaks" due to yeasts. The development of resistance may have biased our data not only for yeasts but also for other microorganisms.

There was a gradual decrease in the number of "breaks in isolation" per monkey as treatment continued (Table 2.VIII), while the relative number of colonizations that disappeared spontaneously increased. The handling of the monkeys in the weeks after the first week of treatment was unchanged. It is possible and even probable that a number of "breaks in isolation" were in fact escapes from antibiotic suppression. These observations led to the conclusion that complete bacteriological sterility as in germfree animals is seldom obtained in monkeys during a short period of treatment. Therefore, when all

cultures of the samples from a monkey are sterile, all that is achieved is an extreme suppression of the microflora, not a germfree condition. Only after prolonged treatment as described for mice by Van der Waaij and Sturm (1971a) and confirmed by Srivastava et al. (1976) might complete sterility be obtained.

When a positive culture was unexpectedly obtained from a previously "negative" sampling area, it was impossible to decide whether this was the first of a series of positive cultures (a colonization) or just an incidental isolation (a contamination). This matter will be discussed more extensively in Chapter V.

Due to the relative resistance of anaerobes to cephalosporin antibiotics and aminoglycosides, these agents are not considered to be drugs of choice for therapy of infections due to anaerobic microorganisms (Martin et al., 1972). However, neither in the Gram-stained smear of the faeces nor in the thioglycolate broth culture was an indication found that microorganisms were present. The antibiotic concconcentration of the intestinal contents may have been sufficient to suppress these anaerobes (Chapter VII); however, had more sensitive technics been applied, small numbers of anaerobes might have been found.

Evaluation of the efficacy of the sensitivity test applied seemed not warranted. The microflora of the monkeys was found to be so sensitive in this test that there was a free choice between the aminoglycosides tested. The minor differences in the sensitivity of the faecal flora expressed as difference of growth in one cup can be neglected, since the faecal concentration after oral administration of the antibiotics was found to be many times the minimum bactericidal concentration as determined in the test (Chapter VII).

It was felt to be inappropriate to express the efficacy of the microflora suppression as per cent sterile cultures obtained as has been done by, e.g., Levi et al. (1973) and Levine et al. (1973). A low grade colonization does not lead to positive cultures each day. This gives a false impression of the quality of the decontamination, since too many "negative days" would be included. Furthermore, colonization by a microorganism that becomes invasive leading to a fatal infection of the monkey may result in only a few days with positive culture(s) but is of much greater significance than a prolonged colonization with a noninvasive microorganism. Finally, the number of days with positive cultures can be influenced by the experimenter's decision as whether or not to adjust the antibiotic treatment immediately or only after some days. The latter, for example, was sometimes done in the present study with otherwise healthy monkeys. For the same reason, the interval during which a monkey was colonized is not presented.

In about 50% of the monkeys, complete microflora suppression was achieved at the end of the treatment period considered in this chapter. The microorganisms present were mainly yeasts and non-fermenting Gram-negative rods. The consequences of their presence during immunosuppression will be discussed in Chapter IV.

CHAPTER III

SYSTEMIC SIDE-EFFECTS OF DECONTAMINATION

3.1 INTRODUCTION

In gastrointestinal decontamination, nonabsorbable antibiotics are administered orally. The side effects of the regimen can be the results of a direct toxic effect of the agents applied but can also be due to the suppression of the microflora. Removal of the microflora may have a dramatic effect on the normal physiology of the individual in general and on the gastrointestinal tract in particular. Despite much work - performed mainly on rodents - the basic mechanisms remained unknown but alterations in many parameters were described. An example of a direct effect of neomycin was described by Thompson et al. (1972) in germfree pigs. Administration of neomycin resulted in increased faecal excretion of endogenous neutral sterols and fat. The bile excretion remained unchanged and no damage was found in the intestinal lining. Absence of the microflora can result in accumulation of several substances that are normally metabolised by the microflora, for instance, β-aspartylglycine (Welling and Groen, 1978). These non-metabolised substances might exhibit toxic effects (Gordon and Pesti, 1971). Furthermore, the oxidation-reduction potential has been found to be much higher in germfree rodents as compared to conventional controls (Ruseler-van Embden, 1975). In plasma, Fe and Cu levels were found to be somewhat lower in germfree than in conventional rats. The liver of the germfree rat stored more, but the other organs contained less iron than their conventional counter parts. The haematocrit and haemoglobin levels were comparable in both groups (Reddy, 1965). The same author also reported an increased absorption of calcium and magnesium in germfree animals in comparison with their conventional controls (Reddy, 1972).

The enlarged caecum in germfree rodents was noted as early as 1896 (Nuttall et al., 1896). A similar enlargement of the caecum develops in rodents during decontamination (Van der Waaij, 1969). The enlarged caecum itself also has profound effects on the animal's physiology. For instance, the cardiac output in germfree rodents has been reported to be 2/3 that of conventional animals (Gordon et al., 1963); caecumectomy results in normal values (Wostmann et al., 1968). No similar

caecal enlargement is seen in primates after decontamination (unpublished observations). The rhesus monkey develops a mild diarrhoea during antibiotic decontamination (Chapter II). It is unknown whether this diarrhoea is due to irritation of the bowel by the antibiotics, (osmotic effects of) undegraded intestinal mucus or bile salts, changes in oxidation-reduction potential, pH or by substances which are otherwise metabolized by the microflora.

In dogs, after oral administration of neomycin, increases in free and total acidities, peptic activity and chlorides of gastric secretions have been reported. In these animals, decreases in serum glucose, chloride and sodium levels were observed (Modi et al., 1979). Jacobson et al. (1960) found in human volunteers that, after oral administration of neomycin, the plasma carotene concentration decreased, indicating decreased fat absorption. In addition, they found a decreased absorption of Iron-59, a decreased urinary excretion of vitamin B_{12} and of D-xylose. They also found a lowered glucose tolerance curve and a decrease in serum cholesterol. In decontaminated patients, Cohen et al. (1976) observed a decrease in serum carotene and folate, an impaired absorption of D-xylose; fat globules and muscle fibers were demonstrable in the stool, indicating defective digestion.

In healthy monkeys, the diarrhoea generally does not affect the animal's condition and does not result in dehydration, as it does in irradiated monkeys (Chapter VI). The antibiotics administered for gastrointestinal decontamination, particularly neomycin, are nephro-and ototoxic and may cause neuromuscular blocking after parenterial administration (Lechevalier, 1975). Because these so-called nonabsorbable antibiotics are minimally absorbed, there is a potential danger of toxicity. In human pathology, it is known that about 3 % of neomycin is absorbed from the gut (Waisbren, 1976), which, in severe renal impairment and dehydration, can be enough to cause toxic effects. To detect toxic effects, it was decided to monitor a number of haematological and biochemical parameters longitudinally during antibiotic decontamination so as to investigate the occurrence of toxic side-effects of the decontamination procedure on bone marrow, liver-and kidney functions.

3.2 MATERIALS AND METHODS

3.2.1 Monkeys

Of the 53 Macacca mulatta (rhesus) monkeys submitted to gastrointestinal decontamination as described in Chapter II, blood samples

were taken from a superficial leg vein at a frequency of 2 ± 1 sample per week. The samples were taken in the morning hours before any oral treatment was given. The body weight was determined each morning before any food was administered.

For haematologic determinations, 10 droplets of blood were collected in a 2 ml vial containing a few crystals of versene (dipotassium salt of diamino-ethane-tetraacetic acid. 2 H_2O) to prevent clotting. The haematocrit was determined by a capilary tube method, the leukocytes were counted with a Coulter counter and the thrombocytes were counted according to the Feissly method (1949). The differential count was performed on a Giemsa-May Grünwald-stained blood smear. The reticulocyte count per 1000 erythrocytes was determined in a brilliant cresyl blue-stained smear.

The biochemical tests were performed on a serum sample derived from approximately 3 ml blood. They were performed in the biochemistry section of the Pediatric Department of the Academic Hospital, Leiden University (Dr. M.A.H. Giesberts).

3.2.2 Interpretation of data

The data were divided into four groups according to the treatment phase. The groups were:

a) data obtained before antibiotic treatment;
b) data obtained from day 1 to day 6 (the high dose period);
c) data obtained from day 6 to day 15 ("adaptation period");
d) data obtained from day 15 to the end of the treatment (irradiation or discontinuation of antibiotics).

The range of the values obtained was subdivided into small intervals. The data were plotted as a percentage of the total number of observations separately for each group defined above.

To investigate the effect of the complete absence of microorganisms on the leukocyte and differential counts, a subpopulation of 12 "clean monkeys" was selected. These animals were selected on the basis that no colonization of any body site was observed.

3.3 RESULTS

The absolute numbers of determinations per treatment period are presented in Table 3.I. The small fluctuations in the number of deter-

Table 3.I

NUMBER OF DETERMINATIONS IN THE TREATMENT GROUPS AS PRESENTED IN FIGURES 1-14

Test	Before treatment	Determinations day 1-6	day 6-15	> day 15
Haematocrit	52	57	116	156
Thrombocytes	54	55	123	158
Reticulocytes	44	39	70	104
Leukocytes	47	52	116	159
% Mononuclear cells	53	51	113	162
"Clean" monkeys:				
1. leukocytes	13	13	27	19
2. % mononuclear cells	11	13	28	17
Sodium	55	60	123	169
Potassium	50	60	115	161
Chloride	55	57	119	161
Calcium	50	59	121	147
Urea	49	47	106	141
Protein	49	59	110	155
S.G.P.T.	19	18	41	43

minations that were performed simultaneously are due to rejection of data due to, for instance, haemolytic sera. The number of serum glutamate pyruvate transaminase (S.G.P.T.) determinations is limited because a sufficient amount of serum was not always available.

As described in Chapter II, the number of monkeys available for observation decreased after day 15, because many animals were irradiated.

The haematological values are presented in Figs. 3.1, 3.2, 3.3, 3.4 and 3.5. During decontamination, the haematocrit decreased about

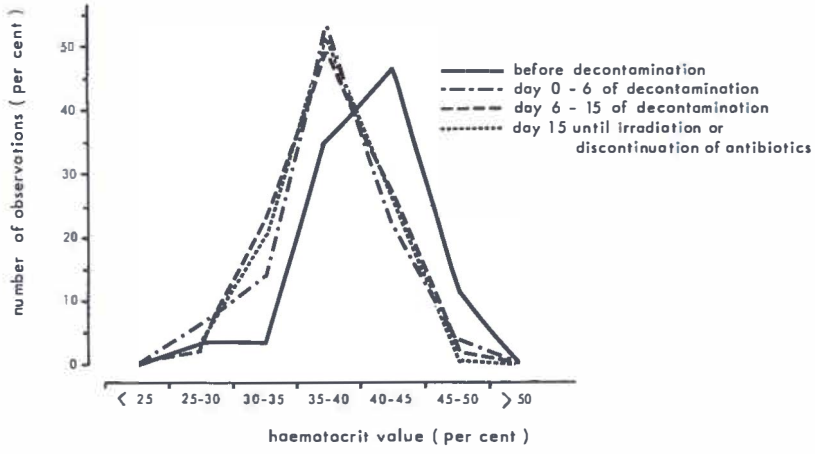

Figure 3.1:
Per cent haematocrit (before and during several periods of decontamination).

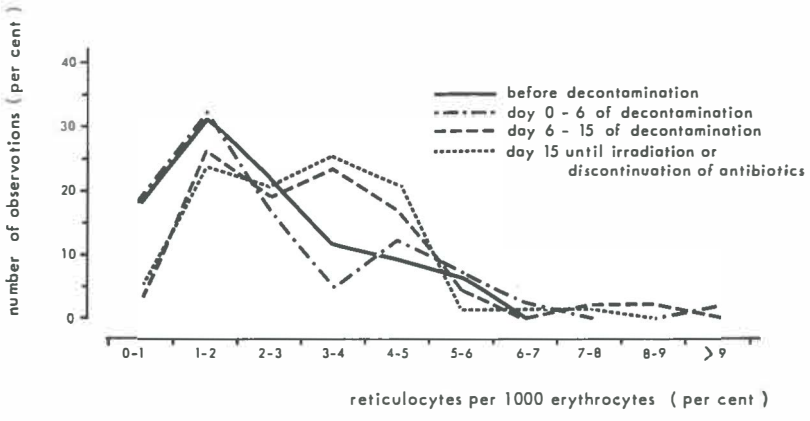

Figure 3.2:

Per cent of reticulocytes of erythrocytes (before and during several periods of decontamination).

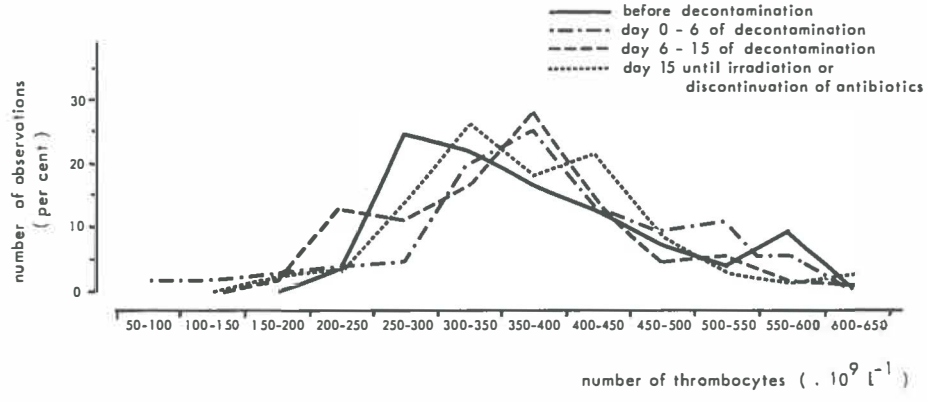

Figure 3.3:

Number of thrombocytes (before and during several periods of decontamination).

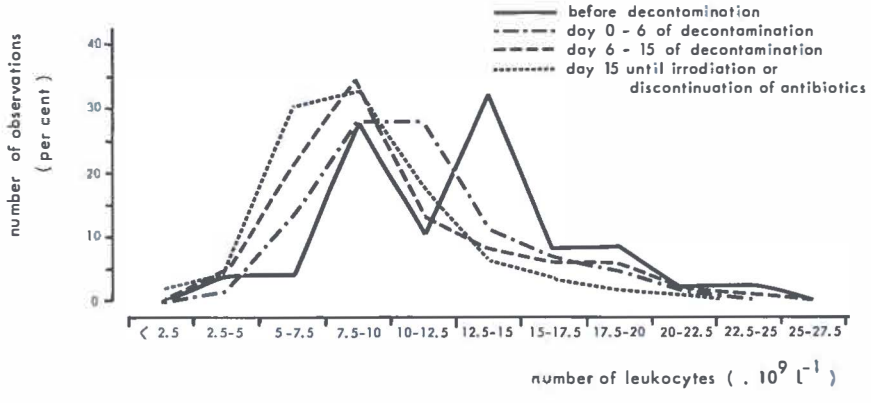

Figure 3.4:

Number of leukocytes (before and during several periods of decontamination).

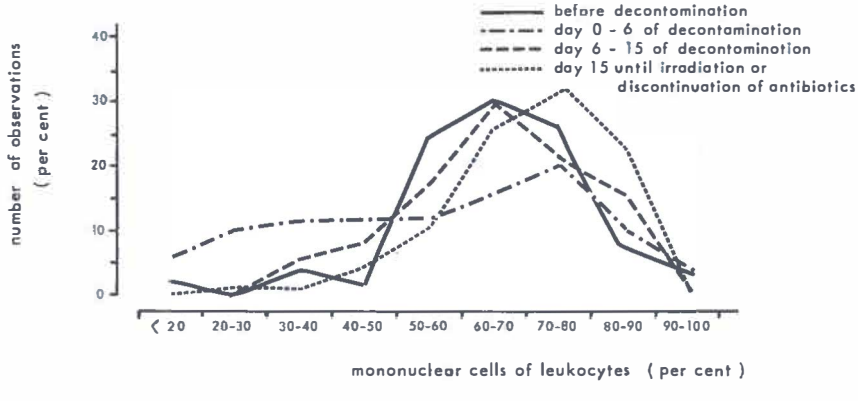

Figure 3.5:

Per cent of mononuclear cells of leukocytes (before and during several periods of decontamination).

5%, while all other haematological values studied remained unchanged. Note that, also in the "clean" monkeys, the number of leukocytes and the differential count remained unchanged (Figs. 3.6 and 3.7).

With regard to the electrolytes, sodium (Fig. 3.8) and potassium levels (Fig. 3.9) appeared stable, while the chloride level (Fig.3.10)

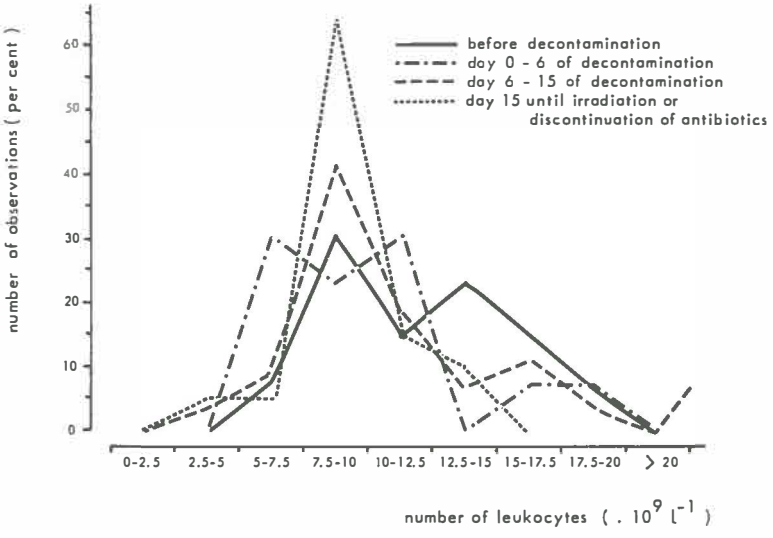

Figure 3.6:

Leukocytes in "clean" monkeys (before and during several periods of decontamination).

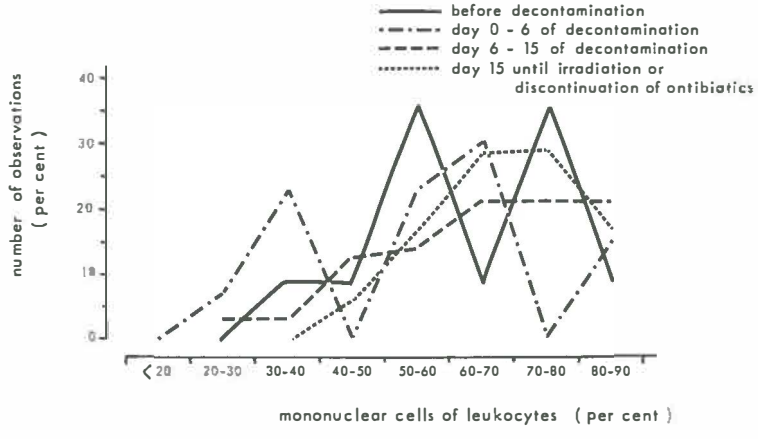

Figure 3.7:

Per cent of mononuclear cells of leukocytes in "clean" monkeys (before and during several periods of decontamination).

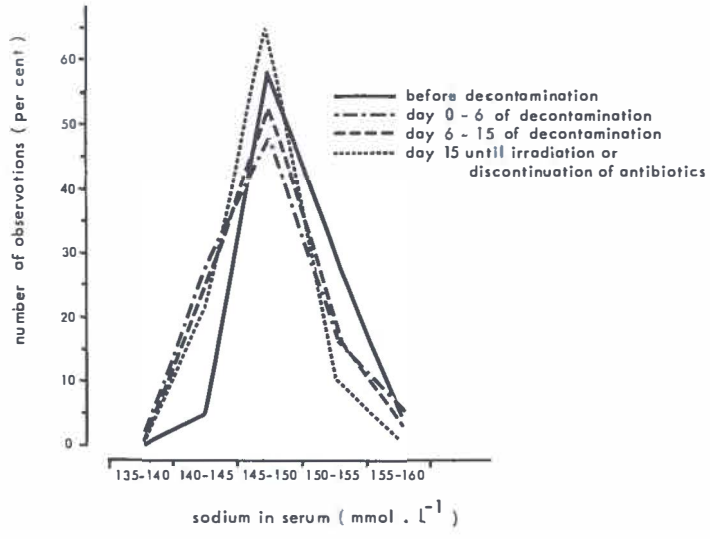

Figure 3.8:

Sodium in serum (before and during several periods of decontamination).

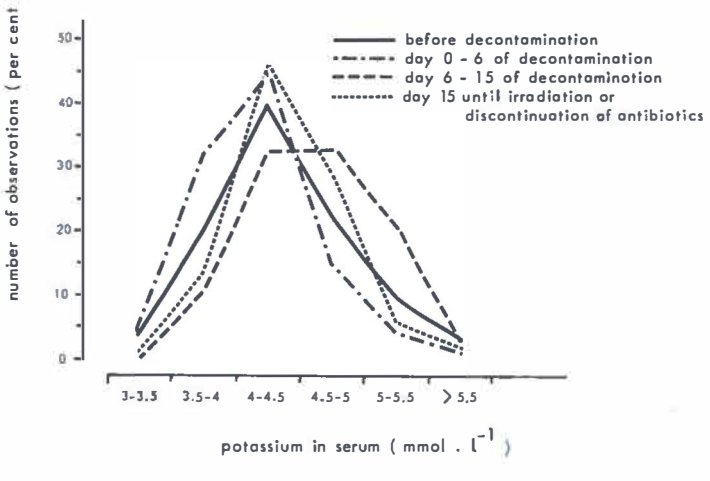

Figure 3.9:

Potassium in serum (before and during several periods of decontamination).

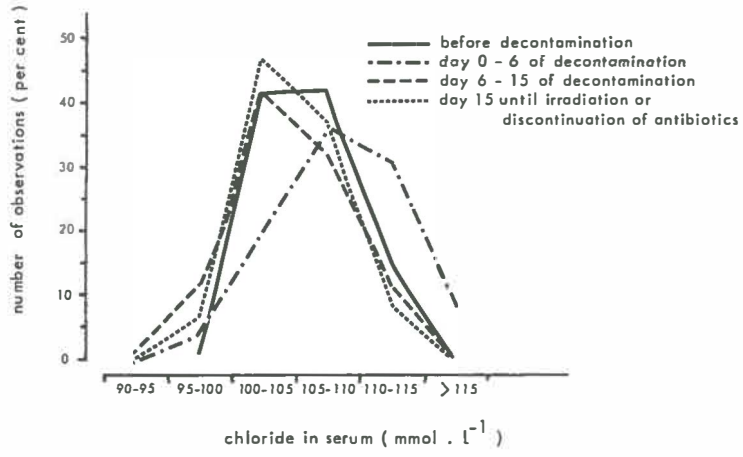

Figure 3.10:

Chloride in serum (before and during several periods of decontamination).

appeared to increase somewhat in the first days of decontamination. The serum calcium concentration showed a slight tendency to decrease in these first days (Fig. 3.11). Serum urea concentration also increased in the first days of decontamination, but subsequently returned to normal values (Fig. 3.12). The serum protein concentration (Fig. 3.13) and the S.G.P.T. level remained constant (Fig. 3.14). A high value for the S.G.P.T. was found in one monkey. However, these increased S.G.P.T. values returned to normal during the following 7-14 days without any change in therapy.

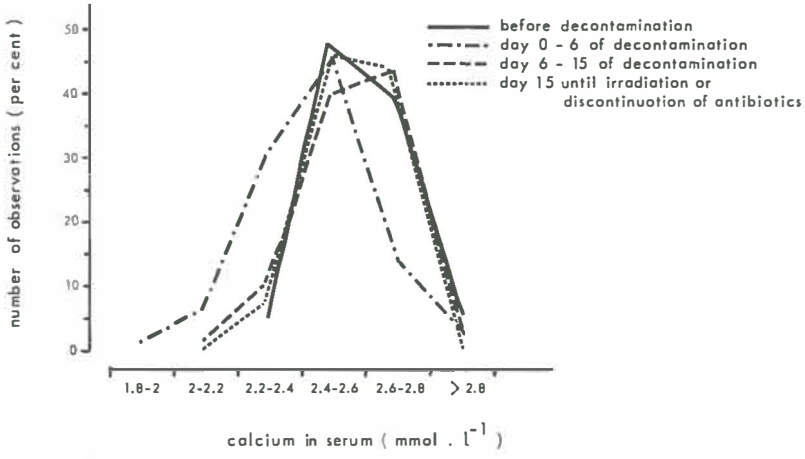

Figure 3.11:

Calcium in serum (before and during several periods of decontamination).

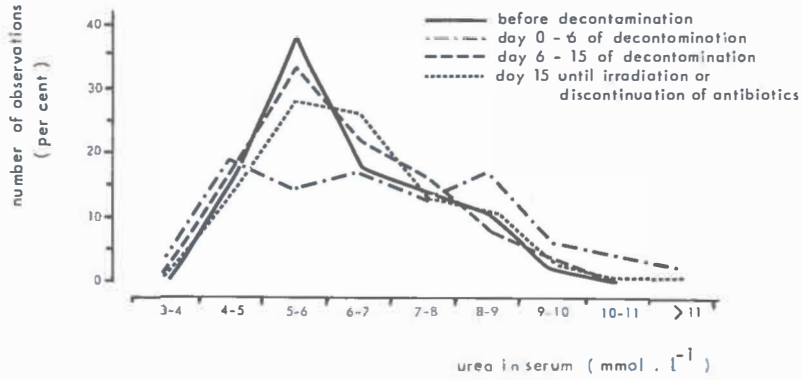

Figure 3.12:

Urea in serum (before and during several periods of decontamination).

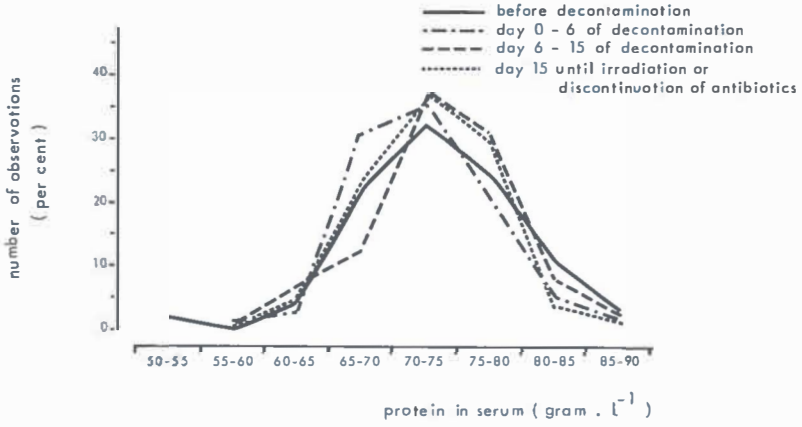

Figure 3.13:

Protein in serum (before and during several periods of decontamination).

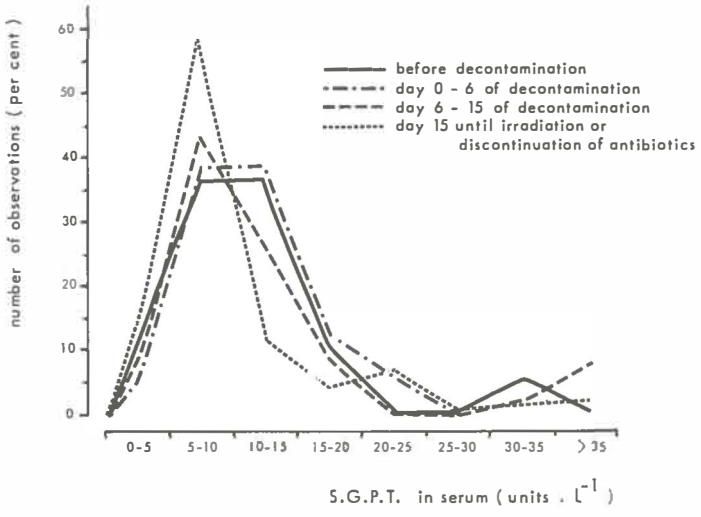

Figure 3.14:

S.G.P.T. in serum (before and during several periods of decontamination).

3.4 DISCUSSION

Decontamination of the digestive tract with "nonabsorbable" antibiotics can be safely performed in healthy monkeys. Despite several obvious alterations in the physiology of the gastrointestinal tract, the monkeys thrived well after a short period of adaptation. Even if decontamination was continued for prolonged periods (up to 72 days), no toxic side-effects were seen.

In the first days of decontamination, the majority of the monkeys became ill and some vomited. There was generally a reduction in food intake (Chapter II). This may explain the increase in urea and chloride levels in the serum in this period. In contrast, the haematocrit decreased during the same period. This cannot be explained by a simple retention of water and electrolytes, since no weight gain was observed in this period; however, this may have been masked by loss of body reserves due to the reduced intake of food. The decreased haematocrit value was not restored in the subsequent treatment periods considered here and appears therefore to have been basically related to the decontaminated state of the monkey. The observed changes in urea, chloride and haematocrit could also be due to a shift of water from extra-intestinal sites into the intestine due to accumulation of products leading to increased osmotic pressure. This could also explain the diarrhoea.

The somewhat reduced calcium levels of the serum in the first days of treatment might be explained by the decreased food intake as well as by binding of the calcium to the sulphate group of the neomycin in the intestine . When the mentioned alterations in fat metabolism also occured in the monkeys, the lost calcium could have been bound to the fats. Clinical signs of calcium depletion such as tetanic seizures were never observed. In the period following the first treatment period of one week, a new equillibrium resulting in normal values had apparently been established.

For monitoring the glomerular kidney function, the creatinine clearance is generally regarded as more appropriate than the urea concentration in the serum. It was considered impossible to collect all urine produced in 24 hours. The amounts of blood required for determinations of the serum creatinine were also too large to collect from a monkey weighing between 1800 and 3000 grams at regular intervals. Since all fluctuations in serum urea concentration correlated with the hydration status of the monkey and since in no case were permanently increased values observed, it could be concluded that no irreversible toxic damages to the kidney had occurred in our series, which could have made the decontamination procedure hazardous.

Although all animals developed soft faeces, they could apparently compensate for the loss of water and electrolytes quite well. Potassium chloride was added to the liquid food in a dose of 5 $mg.ml^{-1}$ not for therapeutic reasons but to accustom the monkeys to its taste before irradiation. In the preirradiation period, the monkeys thrived just as well when the potassium supplementation was omitted (previous unpublished observations). After irradiation (Chapter VI), a rather heavy potassium loss had to be compensated for.

Van der Waaij et al. (1970) reported that, in 3 decontaminated monkeys in the first 2-3 weeks of decontamination, a decrease in the granulocyte count and a relative increase in the lymphocyte count occurred. The total number of leukocytes showed a tendency to increase in the first days of treatment, but decreased thereafter to pretreatment levels in these 3 monkeys. The data of our study do not confirm these observations. No changes in any blood cell count during antibiotic decontamination were found.

As described in Chapter II, a number of monkeys continued to be colonized during antibiotic decontamination. In these animals, the presence of microorganisms might have stimulated leukocyte production by induction of colony stimulating factor (MacVittie and Walker, 1978). The leukocyte and differential counts were therefore studied in monkeys that were never colonized in the treatment period considered.

Also in these "clean" monkeys no effect on the differential count was seen during treatment, although the total number of leukocytes showed a slight tendency to decrease after the first 6 days of treatment. The thrombocyte and reticulocyte counts remained unchanged. This may indicate that antibiotic decontamination is not toxic for the bone marrow.

Decreased numbers of leukocytes and erythrocytes (resulting in a decreased haematocrit) have also been described in germfree beagles (Yale and Balish, 1976), but was not found in germfree rats (Reddy et al., 1965). In decontaminated dogs a slightly decreased leukocyte count (Hayes et al., 1974) as well as a reduced granulopoietic stimulation (Walker et al., 1978) was found. In both publications, no effects on other haematological and biochemical parameters were mentioned. Since the determinations were done less frequently in these dogs than in this study, minor fluctuations in the initial phase of the decontaminations may have gone unnoticed.

CHAPTER IV

MAINTENANCE OF MICROFLORA SUPPRESSION IN IRRADIATED MONKEYS

4.1 INTRODUCTION

Infections frequently develop in patients submitted to bone marrow transplantation (Clift et al., 1974; Thomas et al., 1975; Kenny and Hitzig, 1979; Winston et al., 1979). To reduce the exogenous microbial pressure on these transplanted patients, they are submitted to isolation in laminar air flow rooms (De Koning et al.,1970; Solberg et al., 1971). As a preclinical model, bone marrow transplantation was performed in rhesus monkeys (Van Bekkum et al., 1969; Dicke et al., 1978). In the monkeys described here, prevention of infection was attempted during periods of extreme immunosuppression by application of strict reverse isolation and gastrointestinal decontamination as mentioned in Chapter II for healthy monkeys. After a decontamination period of at least 2 weeks, there was incomplete suppression of the flora in a number of animals (Chapter II). If Enterobacteriaceae species or Pseudomonas species were detected, the irradiation was postponed, since the latter are particularly known to become invasive in neutropenic animals (Wensinck et al., 1957; Van der Waaij et al., 1978). The presence of miscellaneous "non-fermenting" Gram-negative rods and yeasts was not considered a contraindication for irradiation, mainly because, in our experience, it was impossible to suppress these microorganisms. It will be discussed whether the assumption that they would not become invasive was correct.

As described earlier (Chapter II), it is often very difficult to completely eradicate the oro-gastrointestinal microflora. It was therefore decided to introduce the term extreme flora suppression. To what extent the microflora could be maintained suppressed is discussed in this chapter.

4.2 MATERIALS AND METHODS

4.2.1 Monkeys

Fifty-five Macaca mulatta (rhesus) monkeys were obtained from the Primate Center TNO. They were decontaminated as described in Chapter

II. Forty animals were taken from the group described in Chapter II; another 15 were treated with nalidixic acid before the decontamination procedure was started. By pretreatment with nalidixic acid, it was aimed to eliminate the Enterobacteriaceae without suppression of the other (anaerobic) components of the microflora (Van der Waaij and Berghuis-de Vries, 1974a). The results of these experiments are not discussed here.

4.2.2 Isolation procedure

Isolation was in peracetic acid sterilized laminar air flow cabinets, as described in Chapter II.

4.2.3 Food and drinking water:

The composition and the method of administration of the food and water was as described in Chapter II. However, sodium and potassium chlorides were added as required (Chapter VI) to compensate for loss of electrolytes due to diarrhoea. At the end of the decontamination period and if the monkey was in a clinically good condition, small amounts of autoclaved grain and food pellets as well as sterilized Liga® were made available to the monkeys.

4.2.4 Bacteriological procedures

Sampling sites and the frequency of sampling were the same as described in Chapter II. When the leukocyte count was below 1.0×10^9 cells.l^{-1} blood, 3-5 droplets of blood were cultured in thioclycolate broth daily. These "blood cultures" were started during the study. A monkey was considered to be colonized with a particular microorganism when the same species was isolated at least twice from either of the sampling sites within a period of 7 days. A "break in isolation" was thought to have occurred when the microorganism was not isolated beforehand from that monkey. Reappearence of a microorganism was defined as a colonization by a microorganism which had been suppressed for a period of more than 7 days.

*Biscuits made by Liga B.V., The Netherlands.

4.2.5 Irradiation procedure

Inside the laminar air flow bench (L.F.), the monkey was placed into a plastic cylinder with air holes that had been presterilized with 2% peracetic acid. This cylinder was double-wrapped with sterile cloth and taken out of the isolator and transported to the irradiation source. All monkeys were submitted to 8.5 Gy of X-rays, 300 kV H.L.V. 3 mm Cu, distance source to centre of the cage: 0.65 m. During the irradiation, conditions of maximal back scatter were maintained and the monkey was rotated so as to obtain an equal distribution of the X-rays over the body. After irradiation, the outer layer of cloth was taken off in front of the L.F., the inner layer was taken off inside the L.F. and the monkey was put back into its cage.

4.2.6 Bone marrow grafting

The animals were transplanted with bone marrow twenty-four hours after irradiation. Nineteen monkeys received an autologous graft (6×10^7 cells.kg^{-1} body weight); 29 received an allogeneic graft obtained from a random nonrelated donor (5×10^7 cells.kg^{-1} body weight) and 7 were not grafted. All grafts were provided by the bone marrow transplant group of our institute (Dicke, Löwenberg, Wagemaker).

4.2.7 Supportive treatment

When the platelet count fell below 20×10^9.l^{-1}, platelet transfusions were given. When required, erythrocyte suspensions were also given. All cells for transfusions were obtained from unmatched donors. To prevent undue Graft versus Host (GvH) reactions (Groff et al.,1976; Ford et al., 1976), all transfusion suspensions were irradiated with 10 Gy. When the biochemical parameters* (Chapters III and VI) indicated shortage of serum water and electrolytes, these were provided not only as additions to the food but also subcutaneously or intravenously.

Since a haemorrhagic tendency that responded to administration of vitamin K was observed in one monkey after irradiation all subsequent monkeys received once a week 1 mg Konakion^{R}** intramuscularly after irradiation if the thrombocyte count was above 20×10^9.l^{-1}. Vitamin K deficiency has been described in germfree animals (Luckey, 1963b).

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*as determined in the Biochemistry Section of the Department of Pediatrics of the Leiden Academic Hospital (Dr. M.A.H. Giesberts).

**Hoffmann-Laroche. Vitamin K_1

The observation period after irradiation was divided into:

a) the first period of 10 days, in which the bone marrow destruction became evident;
b) a second period of 10 days, in which supportive treatment with blood products was obligatory;
c) a third 10-day period, in which the engrafted or the animals own bone marrow resumed its activity;
d) a 20-day period in which the bone marrow function was further restored;
e) a period after day 50 following transplantation up to termination of decontamination. In this period, the defense capacity was restored to some extent.

4.3 RESULTS

4.3.1 Clinical results

Whole body irradiation has the side-effect that it makes the animals ill. Many monkeys vomited during the first 24 hours after irradiation. With a few exceptions, the animals did not take the antibiotic containing food spontaneously. They were therefore nourished by forced feeding through a stomach tube.

The consistency of the faeces changed dramatically after irradiation, from soft to a watery substance with some solid particles. This obviously led to dehydration and electrolyte loss. The data obtained by Korthals Altes (1971) indicated a severe hypopotassemia. Therefore, up to 30 ml a day of a 10% KCl solution was added to the food to maintain an adequate potassium serum level (Chapter VI). When a low sodium content of the food was recognized, sodium supplementation was also given (Chapter VI).

4.3.2 Bacteriological results

On the day of irradiation, 22 out of the 55 irradiated monkeys were colonized at some of the sampled sites. The microorganisms present are presented in Table 4.I. In 4 colonizations, the day of irradiation appeared to have been the last day of colonization. It can be seen in Table 4.VII that 10 colonizations disappeared and 17 persisted, 4 of which were considered to have contributed to the death of the monkey. None of the Gram-positive microorganisms caused infec-

Table 4.I

COLONIZATIONS PRESENT AT IRRADIATION
(in 22 out of 55 monkeys)

Microorganism:	Number of colonizations	Sites involved		
		oral	faecal	skin
Staphylococci	2	2	-	-
Streptococci (excl. Strept. faecalis)	2	2	-	-
Other Gram-positive microorganisms	3	-	1	2
Enterobacteriaceae*	1	-	1	-
Pseudomonas*	1	-	1	-
Other non-fermenting Gram-negative rods	7	7	-	-
Yeast	11	10	1	2
Total	27	21	4	4

*Colonization not known at irradiation.

tions. Particularly worth mentioning is a monkey whose left ear was colonized by a Bacillus species; it was never eradicated, but also never colonized other sampled sites.

The faecal cultures growing Pseudomonas aeruginosa and E. coli became positive only after irradiation. If their presence had been known at the time of irradiation, the irradiation would have been postponed. The animals died, respectively, of a Pseudomonas aeruginosa septicemia and a septicemia due to E. coli, in conjunction with a Pseudomonas aeruginosa which was initially isolated on the day before death. Seven monkeys were colonized with non-fermenting Gram-negative rods. In the first monkey found to be colonized, this slowly growing microorganism was at first not regarded as potentially pathogenic. Later, treatment was nevertheless adjusted to the sensitivity pattern of the microorganism and streptomycin was replaced by neomycin. However, when all life supportive treatment had to be stopped due to inaccessibility of the veins, the animal died. The non-fermenting Gram-negative rod was isolated from the its heart blood at autopsy. Since the thorax was not opened aseptically, the possibility of contamination during sampling of the blood was probable.

In the next three monkeys from which non-fermenting Gram-negative rods were isolated prior to irradiation, the microorganisms were tested for pathogenicity. Germfree ND mice were associated with this microorganism and irradiated with 8.8^{2} Gy. In none of these mice could

the microorganisms be isolated from the heart-blood culture. With this information, the three monkeys were irradiated and grafted with autologous bone marrow. In none of them did infections occur. These three monkeys fared well even though all samples showed growth of this particular microorganism. Based on this observation, non-fermenting Gram-negative rods (excluding Pseudomonas sp.) were not considered as contraindications for irradiation in subsequent experiments.

One monkey died of a severe GvH reaction. In this animal, colonization with a Klebsiella species was suppressed. However, a yeast remained present in the cultures and colonized all sampled sites. Attempted suppression of the yeasts with amphotericin B or miconazole* failed. The presence of these yeasts had therefore to be accepted. Despite the large number of monkeys that were still colonized with yeasts during treatment, only in the monkey mentioned above, which suffered from GvH disease, was the yeast finally isolated from all sampled sites at the time of the animal's death.

A number of colonizations with microorganisms that had been isolated beforehand from the same monkey occurred. They had been recorded as a colonizing strain that was later suppressed (Table 4.II). Only bacterial species of the family Enterobacteriaceae rapidly colonized the monkeys and caused severe problems. This phenomenon of "reappearance" of microorganisms will be discussed separately in Chapter V.

Table 4.II

NUMBER OF COLONIZATIONS AFTER IRRADIATION BY MICROORGANISMS ISOLATED AND SUPPRESSED BEFORE IRRADIATION ("REAPPEARANCE")

(in 14 out of 55 monkeys)

Microorganism:	Number of colonizations	Sites involved		
		oral	faecal	skin
Staphylococci	2	2	-	1
Strept. faecalis	1	1	-	-
Streptococci	1	1	-	-
Other Gram-positive microorganisms	4	-	2	2
Enterobacteriaceae	5	5	4	4
Yeast	3	3	3	1
Total	16			

*Miconazole (Daktarin®) was kindly supplied by Janssen Pharmaceutics.

Table 4.III

NUMBER OF COLONIZATIONS DUE TO BREAKS IN ISOLATION
(in 25 out of 55 monkeys)

	Postirradiation days				
	0-10	10-20	20-50	≥ 50	Total
Microorganism:					
Staph. aureus	2	4	1	1	8
Strept. faecalis	-	1	1	2	4
Other Gram-positive microorganisms	4	-	-	2	6
Enterobacteriaceae	5	-	-	1	6
Pseudomonas	1	-	1	-	2
Other non-fermenting Gram-negative rods	2	1	1	-	4
"Anaerobes"	2	-	-	-	2
Total	16	6	4	6	32

In the 55 irradiated monkeys during a total exposure time of 1972 days (range $7-10^5$, median 22.5 days) in 25 monkeys 31 colonizations with microorganisms that were not isolated earlier in their treatment were found. Their presence was interpreted as the result of a "break in isolation". The "breaks in isolation" are presented in Table 4.III. The number of monkeys exposed is presented in Table 4.V. The microorganisms introduced by a "break in isolation" were frequently found initially in the oral cavity. They also often colonized skin and faeces (Table 4.IV). The highest incidence of breaks in isolation was seen in the first 10-day period after irradiation (Table 4.V).

When two samples were found to grow a certain microorganism within a period of 7 days, the monkey was considered to have been colonized during the entire interval. This does not differentiate between a colonization of all sampled sites of the monkey and only two positive samples. Therefore, the number of days on which positive samples were obtained is presented in Table 4.VI. Included are 7 "reappearances" of microorganisms which were suppressed after irradiation.

The efficacy of suppression of colonizations during antibiotic decontamination is shown in Table 4.VII and related to the "origin" of the microorganism. No difference was found between the suppression of colonizations that could be related to a "break in isolation" and those that were likely to result from "reappearance". In 43 out of 55 monkeys, a total of 82 periods of colonization were observed. More

Table 4.IV

COLONIZATION OF MICROORGANISMS AFTER A BREAK IN ISOLATION
(in 25 out of 55 monkeys)

Microorganism:	Number of breaks	Sites involved Oral	Faecal	Skin
Staphylococci	8	6	3	5
Strept. faecalis	4	4	4	4
Other Gram-positive microorganisms	6	4	-	4
Enterobacteriaceae	6	6	4	4
Pseudomonas	2	1	2	1
Other non-fermenting Gram-negative rods	4	3	2	3
Anaerobes	2	-	2	-
Total	32			

than half of these were of limited duration and in only 8 monkeys were 9 microorganisms present at all sampled sites and might have contributed to the death of the monkey.

Table 4.V

NUMBER OF MONKEYS, EXPOSURE TIME AND BREAKS IN ISOLATION

	Total	Treatment period in days 0-10	10-20	20-50	≥ 50
Number of monkeys alive at beginning of the period		55	48	36	17
Number of exposure days in treatment interval	1972	537	386	668	381
Number of breaks in isolation	32	16	6	4	6
Chance for a break in isolation per day	0.016	0.030	0.016	0.006	0.016

Table 4.VI

NUMBER OF DAYS A COLONIZING MICROORGANISM WAS ISOLATED

Microorganism:	Number of colonizations	Number of days 1	2	3	4	5	6	7	≥ 8
Staphylococci	13	6	4	1			1		1
Strept. faecalis	6		1	2					3
Other Streptococci	3	1			2				
Other Gram-positive microorganisms	14	1	7		2				4
Enterobacteriaceae	13			4	2	1		2	4
Pseudomonas	3			1	1				1
Other non-fermenting Gram-negative rods	13	2	1	1					9
Anaerobes	2					1	1		
Yeast	25	3		3					9
Total	82	13	13	12	7	2	2	2	31

Table 4.VII

DEGREE OF SUCCESS IN ELIMINATION OF COLONIZING MICROORGANISMS

	Total	Present at irradiation	"Reappearance"	"Break in isolation"	"Reappearance" of a colonization suppressed after irradiation
Total	82	27	16	32	7
Disappeared without treatment	28	6	9	8	5
Disappeared after treatment	15	4	3	6	2
Died heavily colonized	9*	4	2	3	
Colonization persisted	30	13	2	15	

*Including 1 monkey with 2 colonizations at death (E. coli and Strept. faecalis)

4.4 DISCUSSION

In 55 monkeys submitted to a lethal total body irradiation with 8.5 Gy, 82 periods of colonization by a microorganism were observed. These colonizations could be related to their origin: 27 were present at irradiation, 16 were "reappearances" from before irradiation, 32 resulted from "breaks in isolation" and 7 "reappearances" were recorded after suppressive treatment in the postirradiation period. One-third of these colonizations disappeared without any change in therapy. For these microorganisms, the environment with its antibiotic content was probably too hostile (Chapter VII). Only 8 monkeys died heavily colonized by microorganisms which could not be suppressed with the oral nonabsorbable antibiotics available.

Due to the illness in the first days after irradiation, the total amount of antibiotics taken per day was reduced. Although the faecal antibiotic concentration was not reduced (Chapter VII), the local concentration in the upper part of the digestive tract, particularly in the oral cavity, could quite possibly have been greatly reduced. This could explain the large number of "breaks in isolation" observed in the first period of 10 days after irradiation compared to the number of breaks in the second period. The immunosuppression in conjunction with the reduction in the daily antibiotic intake may have provided favourable circumstances for previously suppressed microorganisms to escape from this suppression and appear at the sampled sites.

Inadequate antibiotic intake not only increased the "endogenous" colonizations but also left the animals less well protected from sensitive exogenous microorganisms. Particularly during this critical period is strict reverse isolation therefore mandatory. Virtually any microorganism that reaches the monkey and that is not inhibited by antibiotics (due to inadequate levels or due to resistance to the antibiotics applied) will colonize the animal more or less densely. The same situation can occur in human patients, as described by Schimpff et al. (1975). They found that life-threatening infections occurred in 4 of 6 patients who discontinued their nonabsorbable antibiotics while still granulocytopenic.

In the first 10 days after irradiation, there is an increased number of "breaks in isolation" compared to the later periods. It is improbable that the increased number of manipulations inside the isolator led to this relatively large number of breaks in isolation compared to the first 10 day period, as, in the second 10-day period in which stomach tube feeding was also applied, even more handling of the animals was required for life supportive treatment such as, for in-

stance, transfusion of blood products. A number of "breaks in isolation" might therefore in fact be "reappearances" of previously suppressed microorganisms.

The immunosuppression by lethal total body irradiation as well as the reduction in antibiotic intake may have enabled microorganisms that were present at irradiation to grow out into high numbers and disseminate to other sample sites for some time. Of the microorganisms present at irradiation, Enterobacteriaceae species and Pseudomonas species should be considered as indications to postpone the irradiation. In those animals where these microorganisms were found to be present, they subsequently became invasive and were isolated from the blood. Similar observations were made in rodents when Pseudomonas species became invasive within one week after irradiation (Wensinck et al., 1957).

When growth occurs in any sample obtained from a decontaminated monkey - and this holds for any sampled site - identification and a sensitivity test should be performed, although not all positive samples will result in a colonization (Chapter V). When the isolate is resistant to the antibiotics applied, the microorganism will not remain at one site but will most probably colonize all sites rapidly. This requires immediate adjustment of the antibiotic treatment. Besides the antibiotics supplied with the food, a high dose treatment regimen of 0.5 grams of an aminoglycoside initially 3, later 2, times daily should be given, if necessary by stomach tube. This treatment schedule should be continued for a prolonged period of time. The microorganism will probably be only suppressed and reappearance may occur easily in those cases.

CHAPTER V

CHANCE FOR AND SIGNIFICANCE OF A POSITIVE SAMPLE

5.1 INTRODUCTION

In gastrointestinal decontamination, the aim is complete elimination of the microflora in order to establish a maximal protective effect. Complete microbiological sterility, however, is seldom achieved. It is better, therefore, to speak of extreme suppression of the microflora, as discussed in Chapters II and IV. In the management of individuals treated with nonabsorbable antibiotics, it is of great importance to be able to predict the significance of a positive culture. Particularly during the period of severe immunodeficiency is it important to know whether the oral antibiotics should be adjusted immediately.

When the anaerobic part of the microflora is eliminated, the colonization resistance is abolished (Van der Waaij et al., 1971b). A microorganism resistant to the antibiotics applied may colonize the monkey in an abnormal pattern and grow out into high densities, which can result in invasion of the body tissues (Van der Waaij et al., 1978).

Faced with a positive culture, the only information that is available within a short period of time is the morphology of the microorganism in the Gram-stained smear. This knowledge is the basis for the data presented in this chapter. One of the major questions is whether to consider the isolated microorganism as a contaminant or merely as the start of a colonization. Since it was found during these experiments (as described in Chapters II and IV) that a colonization often started in the oral cavity, the site of isolation is also to be considered in making the distinction between a colonization or contamination.

It has been stated that "decontamination" leads to selection of multiple resistant strains and should therefore be abandoned (Klastersky et al., 1974). To determine whether this could be substantiated, in our study sensitivity patterns of all major colonizations during decontamination in monkeys were established and will be discussed.

When a microorganism which had colonized a monkey during some phase of decontamination was suppressed, it was of importance to know after which interval the irradiation and bone marrow grafting could be performed with the smallest chance for a reappearance of the suppressed microorganisms. This is of particular importance when microorganisms such as Enterobacteriaceae species or Pseudomonas species, which become invasive in irradiated individuals more frequently, are involved.

5.2 MATERIALS AND METHODS

The bacteriological data obtained in the 40 rhesus monkeys decontaminated as described in Chapter II and irradiated as described in Chapter IV are considered in more detail here. The description of treatment and sampling is given in the chapters mentioned.

A monkey was considered as "colonized" when a particular microorganism was isolated at least twice within a 7-day period. This also included positive cultures from multiple sites for only one day. Left out of consideration were isolations of several species in the first days of the decontamination procedure. A "contamination" was defined as a microorganism isolated from one sample only within a 7-day period. When a microorganism of the same species was isolated again after an interval of more than 7 days, it was considered as a "reappearance". The initial isolation could have been a contamination or a (suppressed) colonization.

Sensitivity testing of the isolated microorganisms was performed by a routine agar diffusion method (Matsen and Barry, 1974). Since the achievable faecal levels of antibiotics are manyfold of the blood levels obtained in systemic therapy (Chapter VII), the critical zones as applied in routine sensitivity testing are not valid. Any zone of inhibition therefore was interpreted as an indication of sensitivity to the specific antibiotic when administered orally.

5.3 RESULTS

In Table 5.I, the initial isolates per sampled site are presented. The percentage of these isolates which were - retrospectively - the start of a colonization is also indicated. When multiple sites became positive on the same day, all positive sites were included in the table. This occurred in 8 monkeys before as well as in 8 monkeys

Table 5.I

INITIAL ISOLATES RESULTING IN "COLONIZATION " AND "CONTAMINATION "
(in 40 monkeys during 1186 pre- and 1444 postirradiation days)

	Gram-positive microorganism			Gram-negative microorganism			Yeast		
		Leading to colonization			Leading to colonization			Leading to colonization	
Preirradiation	Number of isolates	number	per cent	Number of isolates	number	per cent	Number of isolates	number	per cent
oral cavity	20	12	(60.0)	23	18	(78.3)	14	13	(92.9)
faeces	14	6	(42.9)	13	7	(53.9)	2	1	(50.0)
skin	14	5	(35.7)	3	2	(66.7)	3	3	(100.0)
Postirradiation									
oral cavity	23	12	(52.2)	23	17	(73.9)	8	4	(50.0)
faeces	12	3	(25.0)	10	4	(40.0)	4	4	(100.0)
skin	17	10	(58.8)	-	-		4	2	(50.0)

after irradiation. About one half of the colonizations started in the oral cavity before as well as after irradiation. The colonizations outnumbered the contaminations in the oral cavity. The positive samples proved to be the first indication of a colonization in 75.4% of all of the isolates before irradiation and 61.1% after irradiation. Before irradiation, the presence of yeasts was seldom due to a contamination. On the skin sites, Gram-positive microorganisms were more frequently found than yeasts and Gram-negatives. The chance for obtaining a positive sample is presented in Table 5.II.

The results of the sensitivity tests are summarized in Table 5.III. Complete resistance to the antibiotics applied was found in only 3 out of 84 bacterial colonizations. Resistance to cephalosporin was more often encountered: a number of microorganisms such as Enterobacter spp., indole-positive Proteus spp. and Pseudomonas spp. (Simon, 1979) exhibit intrinsic resistance.

As a "contamination", only one microorganism out of 76 was found to be resistant to both antibiotics. The great majority was found to be sensitive.

The time period between the last isolation of a microorganism and day of irradiation was thought to be correlated with its "reappearance" as described in Chapter IV after irradiation in Tables 5.IV and 5.V. When the irradiation was performed within 14 days, 2 out of 4

Table 5.II

CHANCE FOR A POSITIVE SAMPLE *

per 100 exposure days in 40 monkeys during 1186 pre - and 1444 postirradiation days

	Gram-positive microorganism	Gram-negative microorganism	Yeast
Preirradiation period			
oral cavity	1.69	1.94	1.18
faeces	1.18	1.10	0.17
skin	1.18	0.25	0.25
Postirradiation period			
oral cavity	1.59	1.59	0.55
faeces	0.83	0.69	0.28
skin	1.18	-	0.28

*As presented in Table I, this chapter.

Table 5.III

SENSITIVITY PATTERN OF FIRST ISOLATE

	Preirradiation period				Postirradiation period			
	Contamination		Colonization		Contamination		Colonization	
	G^+ mo	G^- mo	G^+ mo	G^- mo	G^+ mo	G^- mo	G^+ mo	G^- mo
A+ C+	1	1	5	2	1		4	1
A+ B+	1		2		3		6	
A+ (B-)		1		2		2	1	6
A+ C-		1	1	8				2
A- C+				1	1			2
A- C-				2		1		1
A+ C not tested				1	1			1
Not tested	23	9	12	7	21	9	12	5
Total	25	12	20	23	27	12	23	18

mo : microorganisms
A : Aminoglycoside
B : Bacitracin
C : Cephalosporine
} + : sensitive, - : resistant
G^+ : Gram-positive
G^- : Gram-negative

Table 5.IV

CHANCE FOR REAPPEARANCE AFTER IRRADIATION OF MICROORGANISMS ISOLATED BEFORE IRRADIATION

(35 contaminants in 40 monkeys)

Microorganism:	Reappearance after irradiation as		Reappearance as a colonization after irradiation (related to the interval between last isolation and irradiation in days)			
	Contamination	Colonization	0-5	5-10	10-15	≥ 15
Staphylococci	1*	1	-/1	-/1		1/2***
Streptococci	1**	-				-/1
Strept. faecalis	-	-	-/1			-/1
Other Gram-positive microorganisms	-	2	1/4	1/1	-/1	-/11
Enterobacteriaceae	-	2	1/1		1/3	-/2
Other non-fermenting Gram-negative rods	-	-		-/1	-/1	-/2
Yeast	-	-				-/1

* Last isolation on day 5 before irradiation
** Last isolation on day 15 before irradiation
***One reappeared as colonization out of 2 contaminations found before irradiation.

Table 5.V

CHANCE FOR REAPPEARANCE AFTER IRRADIATION OF MICROORGANISMS ISOLATED BEFORE IRRADIATION

(29 suppressed colonizations in 40 monkeys)

Microorganism:	Reappearance after irradiation as		Reappearance as a colonization after irradiation (related to the interval between last isolation and irradiation in days)			
	Contamination	Colonization	0-5	5-10	10-15	>15
Staphylococci	-	1				1/2***
Streptococci	-	1	1/2			
Strept. faecalis	1*	-				-/5
Other Gram-positive microorganisms	-	-	-/1		-/1	-/1
Enterobacteriaceae	-	3	1/1	2/4	-/3	-/3
Other non-fermenting Gram-negative rods	1**	-	-/1		-/1	-/1
Yeast	-	1	-/1		1/1	-/1

* Last isolation on day 15 before irradiation
** Last isolation on day 12 before irradiation
***One reappeared as colonization out of 2 contamination found before irradiation

contaminations by these microorganisms reappeared as did 3 out of 8 colonizations, while none of 2 contaminations and 3 colonizations reappeared when the interval was more than 14 days. These differences were not statistically significant ($p > 0.05$).

5.4 DISCUSSION

In this chapter, data are presented on the prospects after a positive culture in relation to the information immediately available such as site of isolation and properties in the Gram-stained smear.

A number of isolates were made only once. These were due to a "contamination" of the monkey itself or of the test system. It is not certain that all of these "contaminations" were indeed of exogenous origin, since some of them "reappeared" as "contaminations" or "colonizations".

In the oral cavity, the number of "contaminations" and "colonizations" was much greater than those obtained from the other sampled sites. As discussed in Chapter II, the oral cavity was found to be a difficult site in which to permanently suppress the microflora. Also the isolation of Gram-positive microorganisms from this site often indicates the start of a "colonization" and their isolation should therefore not be considered as innocuous, although many of these Gram-positives are not as pathogenic as are the Gram-negatives. Since the skin is normally colonized mainly by Gram-positive microorganisms (Marples, 1974; Woodroffe and Shaw, 1974), it is not surprising that initial isolates made at these sites were mainly Gram-positives.

Due to the small number of isolates it is difficult to give a detailed discussion of the significance of a positive sample. There is a good chance that an initial isolate is the first indication of a colonization, regardless of whether it is a Gram-positive or a Gram-negative microorganism. When a yeast is isolated, the chance that one is dealing with a colonization may even be greater. Therefore, if complete microflora suppression is required, there is no justification for a "wait and see policy". From a clinical point of view, however, low numbers of microorganisms of low pathogenicity may be accepted, particularly since these colonizations frequently disappear without further treatment (Chapters II and IV).

Performance of a sensitivity test on each isolate is mandatory. In these experiments, the sensitivity test was sometimes omitted, because cultures became positive only after several days of incubation; therefore, negative cultures of the same site were already available at the

time that the positive one was found. This policy seems not to be justified.

Where sensitivity testing was performed, only 3 microorganisms were not found to be sensitive to the antibiotics applied. However, in a large number of the isolates, no sensitivity test was performed. It is quite improbable, however, that a microorganism resistant to the antibiotics applied would not colonize a monkey for a prolonged period of time. It can be assumed therefore that these microorganisms were sensitive to at least one of the antibiotics applied. Many of these colonizations were of limited duration or intensity (Chapter IV); they disappeared spontaneously. When colonizations were suppressed by adjustment of the antibiotics, quite a number of them reappeared as "colonizations" and a few as "contaminations". This observation again stresses that a germfree state is difficult to obtain by antibiotic decontamination in primates. All that is in fact achieved is an extreme microflora suppression.

Since only 3 out of 84 colonizing microorganisms were resistant to the antibiotics applied, it is not very probable that that oral administration of nonabsorbable antibiotics leads to the induction of resistant strains. However, when the isolation system "leaks" (and it always does to some extent) (Dankert et al., 1978), multiple resistant strains from the environment can colonize the decontaminated individual. If this applies to the situation in hospitals, decontaminated patients can only become SECONDARY foci of multiple resistant strains.

In contrast to Klastersky's observations (Klastersky et al., 1974) in only one case did a Enterobacteriaceae biotype become resistant to the antibiotics applied in the E.O.R.T.C. study (Dankert et al., 1978). The observations in the monkeys are in agreement with this; the bacteria seldom become resistant to the antibiotics applied.

It was impossible to extensively type all microorganisms present in a monkey when the isolation was started. It was reasoned that the finding of two strains of the same species in an isolated monkey was improbable. In the absence of typing facilities, all microorganisms of the same species were therefore considered as identical. Reappearances indicated that a recontamination had occurred from the same source. It was assumed that this source was endogenous, although an exogenous one could not be excluded. However, if exogenous microorganisms were important, a preponderance of some species obtained from the same source would have been expected. This, in fact, occurred in 3 monkeys with non-fermenting Gram-negative rods, as discussed in Chapter II.

When a microorganism was found as a contamination or as a colonization before irradiation the chance for reappearance after irradia-

tion was always present. The interval between last isolation and irradiation seemed not to affect the frequency of reappearances. This again stresses the fact that total eradication is not obtained. It seems prudent, however, to postpone the irradiation for 5-10 days after the last isolation to be sure that the colonization was suppressed, particularly if potentially pathogenic microorganisms are involved.

CHAPTER VI

BIOCHEMICAL PARAMETERS AFTER IRRADIATION

6.1 INTRODUCTION

During gastrointestinal decontamination, the monkeys develop a mild diarrhoea, which does not generally give rise to clinical problems in the healthy animal. No supplementation for lost electrolytes was required before irradiation (Chapter III). After irradiation, however, the diatthoea became severe within a week. Particularly in the three weeks following total body irradiation with 8.5 Gy was the situation critical for the monkeys (Korthals Altes, 1971). During gastrointestinal decontamination of human infants suffering from a combined immunodeficiency, severe imbalances in electrolyte levels and a hypoproteinaemia were found (Vossen et al., 1973). These observations led to the decision to carefully monitor the biochemical parameters in the monkeys.

6.2 MATERIALS AND METHODS

6.2.1 Monkeys

The biochemical parameters as described in Chapter III were monitored two to three times weekly in the lethally irradiated monkeys as described in Chapter IV. According to the haematological "treatment" and its result, the monkeys were subdivided into:

a) 12 monkeys which received no bone marrow graft or an allogeneic graft that failed to take;
b) 8 monkeys which received an autologous graft;
c) 4 monkeys which received an allogeneic graft leading to a take, but did not develop an overt clinical Graft versus Host reaction (GvH);

In early 1974, the monkeys survived the irradiation for only a short period of time, not allowing haematological recovery to take place. These 16 monkeys are taken together as group (d). Only 4 out of these 16 animals lived for more than 20 days after irradiation. In the last group (e), 13 monkeys that were irradiated after group d are described. These monkeys also received sodium supplementation after irradiation.

The two animals which developed a clinical Graft versus Host reaction were excluded, since their clinical symptomatology may have been due more to the GvH reaction than to the decontamination procedure.

As in the healthy monkeys (Chapter III), the biochemical parameters were determined in the Biochemistry Section of the Department of Pediatrics of the Leiden Academic Hospital (Dr. M.A.H. Giesberts).

6.2.2 Interpretation of data

The data obtained were pooled according to intervals: day 0-10, the period in which the effect of the irradiation became evident; day 10-20, the period in which maximal life-support was required; day 20-50, the period of haematological recovery; from day 50 until the antibiotics were discontinued, the period of immunological restoration. The number of observations differed in each treatment interval because a number of monkeys died. Different numbers of observations among several parameters were due to insufficient amounts of serum available. The actual number of determinations is indicated in the figures.

6.2.3 Electrolyte supplementation

Additional KCl was added to the food of all monkeys when the serum values decreased. Guided by the haematocrit, levels of urea, total protein, sodium and chloride and by body weight and the clinical situation, Ringer's lactate (Brocacef) and/or a mixture of 5% glucose and 0.65% NaCl was administered subcutaneously or intravenously; 50-200 $ml.day^{-1}$ were given in 1-4 daily doses.

When it was recognised that the food contained only 82 mg NaCl or 3.57 mMol NaCl per bottle, additional NaCl and sometimes $NaHCO_3$ was added to the food. In these monkeys (group e), the Na^+ and K^+ contents of the urine were determined. The urine collected was freshly voided. In most instances, it was the urine the monkey excreted during caretaking. It was therefore not contaminated with faecal material. The determinations of Na^+ and K^+ were also done at the biochemistry laboratory of the Dept. of Pediatrics, Academic Hospital, Leiden University. The amounts of potassium and sodium in the daily oral intake were increased or decreased in order to establish a urinary concentration of about 100 $mMol.l^{-1}$ for both sodium and potassium.

6.3 RESULTS

Immediately after irradiation, the monkeys were ill; they often vomited during the first postirradiation day. The diarrhoea increased, reaching a maximum after the fifth postirradiation day. At this time, the food intake was so decreased that forced stomach tube feeding was indicated. The maximum amount of food that was tolerated by stomach tube was 50-75 ml four times daily, depending on the body weight.

6.3.1 Sodium (Fig. 6.1).

In groups a, b and c, the sodium content of the serum was 145 mMol on the average. In group c, the sodium tended to increase after day 50. This correlated with the clinical picture; the monkey appeared somewhat dehydrated. Since these four monkeys were also short of breath, no systemic fluid was administered, to avoid lung oedema. In group d, the mean serum sodium was 150 $mMol.l^{-1}$. When the low sodium content of the food was recognized and supplemented for in group e, a dramatic change in the clinical condition of the monkeys occurred; the mean survival increased from 14.6 (median 11.0) days in group d to 22.5 (median 38.5) days in group e. The deaths before day 20 in this sodium supplemented group had a clear-cut etiology, such as septicaemia in 3 monkeys and pancreatitis in one; another died due to haemorrhage.

6.3.2 Potassium (Fig. 6.1).

With the exception of group d, the mean potassium serum value decreased in the postirradiation period. Large amounts of KCl, up to as much as an additional oral intake of 3 gram per monkey, were given. When sodium was added, less potassium had to be given in order to maintain a serum value above 3.5 $mMol.l^{-1}$. Even at serum values below this level, the urinary concentration of potassium was sometimes found to be above 100 $mMol.l^{-1}$. Additional potasium chloride did not increase the serum level but did increase the urinary excretion.

6.3.3 Chloride (Fig. 6.1).

The serum concentration of chloride more or less followed the sodium pattern. In general, additional sodium and potasium chloride was

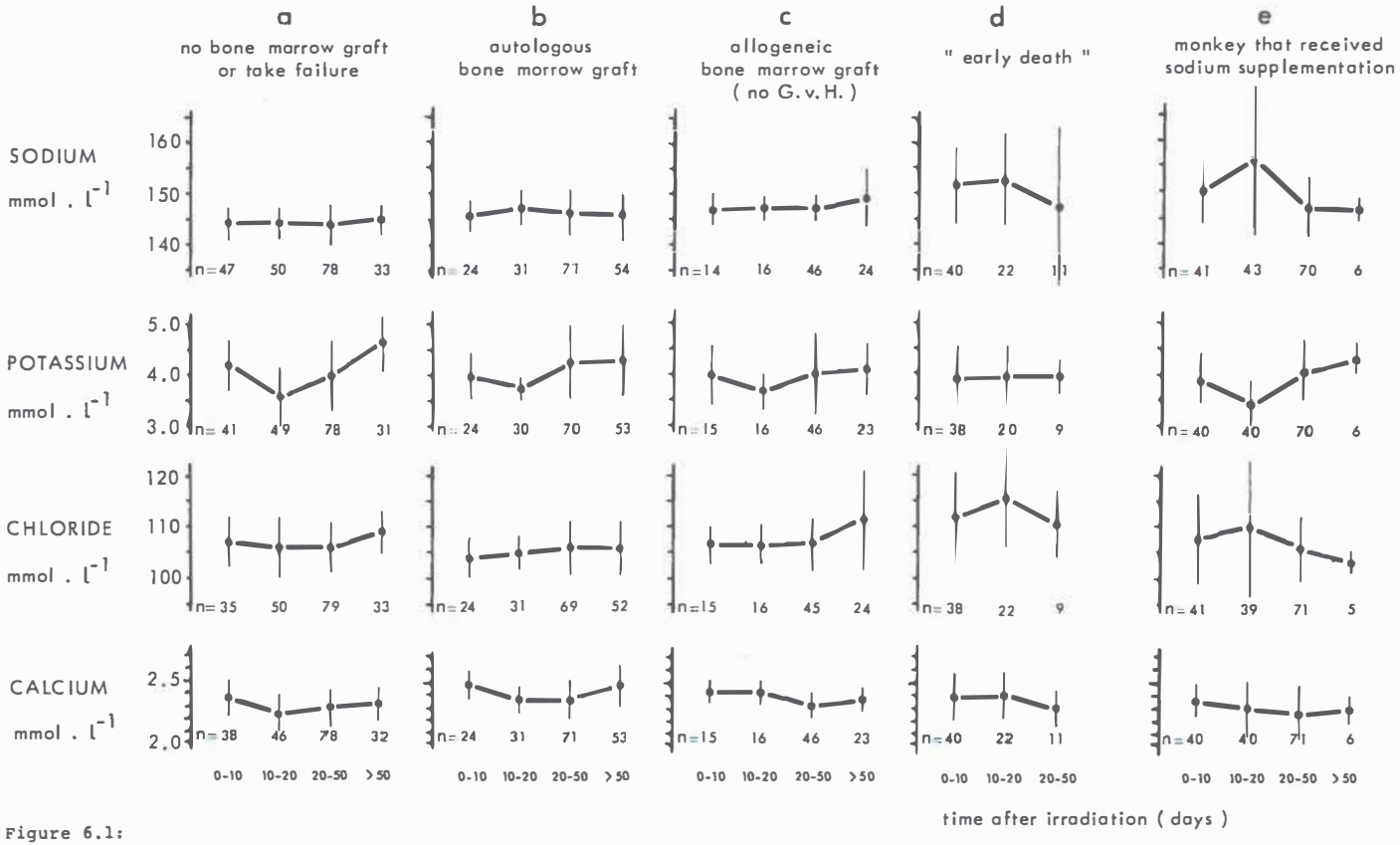

Figure 6.1:

Serum electrolytes in irradiated monkeys. Mean and S D during various intervals in the treatment groups.

required until the bone marrow activity was restored. When this had occurred, the clinical situation was found to be less critical. Many monkeys again took their food spontaneously.

6.3.4 Calcium (Fig. 6.1).

Extremely low values were seldom observed. In a few monkeys, an occasional serum value below 2 mMol.l^{-1} was found. These animals were then treated with calcium gluconate. In all cases, serum values returned to normal. Tetanic seizures were never observed.

6.3.5 Total protein (Fig. 6.2).

The serum protein concentration steadily decreased in all groups after irradiation. After day 50, it tended to stabilize. At serum levels below 55 g.l^{-1}, the monkeys tended to develop oedema of the abdominal skin, which was the lowest point in the favorite resting position of the monkeys when they felt unwell. In some of the animals in which it was investigated, the urine was found to be positive for albumin. This proteinuria, however, disappeared spontaneously.

6.3.6 Urea (Fig. 6.2).

Wide fluctuations in serum urea levels were found. A high serum urea concentration correlated well with dehydration. After rehydration, urea levels returned to pretreatment values. A high urea level was, therefore, not considered as being due to kidney damage.

6.3.7 S.G.P.T. (Fig. 6.2).

Serum glutamate-pyruvate transaminase concentration tended to increase in the postirradiation intervals. No values indicating severe liver damage were obtained.

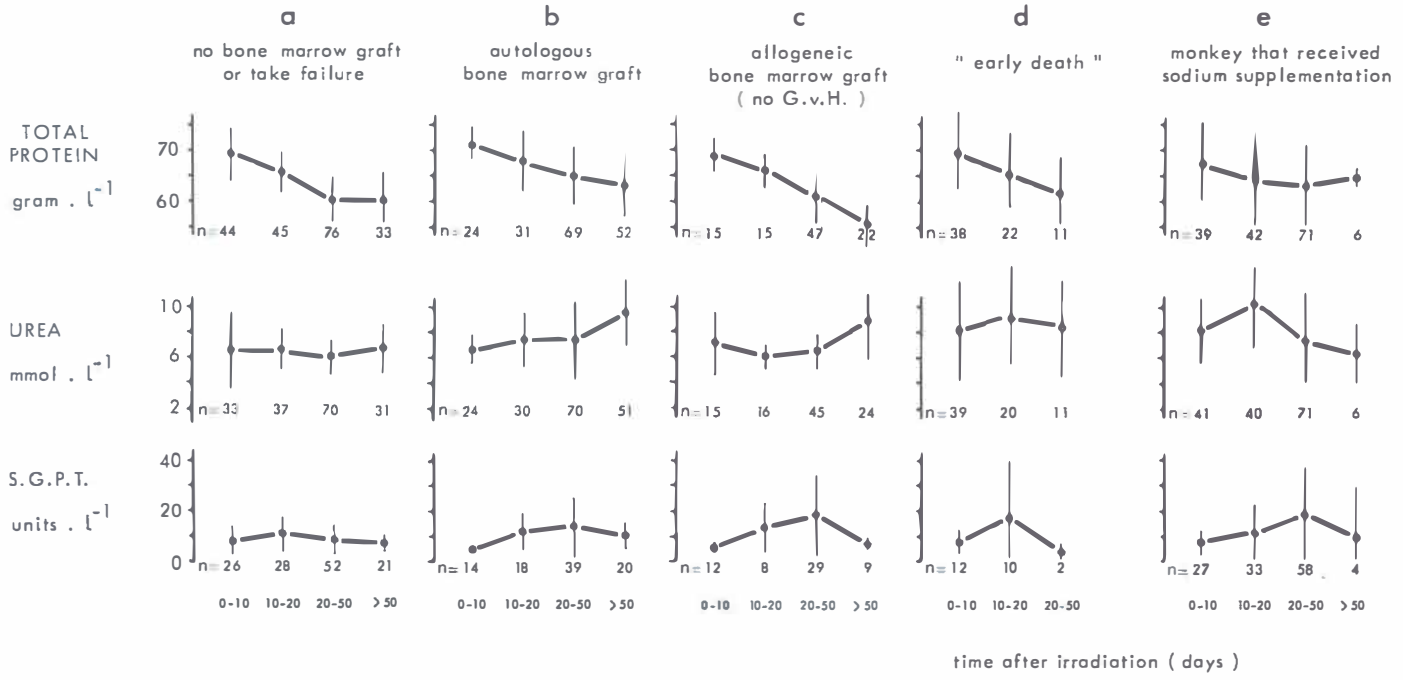

Figure 6.2:

Serum protein, urea and S.G.P.T. Mean and S D during various intervals in the treatment groups.

6.4 DISCUSSION

After the total body irradiation with 8.5 Gy, the very mild diarrhoea (Chapter III) increased in severity in the decontaminated monkeys. This resulted in a significant loss of water and electrolytes, requiring supplementation with potassium and in the later experiments in this study also sodium. The serum protein gradually decreased to levels resulting in oedema in some of the monkeys.

By careful oral supplementation with electrolytes and by stomach tube feeding, the monkeys could be made to survive this critical period. When bone marrow activity was restored, the electrolyte supplements could be discontinued and the majority of the monkeys again took their food spontaneously.

The interval of 5 days between irradiation and evidence of electrolyte imbalances makes an irradiation syndrome of the intestine improbable, since the irradiation syndrome is manifested earlier after irradiation and lesions in general have healed on day 6 after irradiation (Van Bekkum and De Vries, 1967a). Besides, conventional monkeys irradiated with the same dosage on the same X-ray machine in an identical set-up did not develop these electrolyte problems at this time of the experiment (Van Bekkum et al., 1978).

In the monkey model, the supplementation was done mainly by increasing the oral intake. This leaves open the question of uptake from the intestinal tract. It was technically impossible to quantitatively collect the watery faeces and the urine separately; therefore, no further study could be done. An increased oral intake of KCl, however, was followed by an increased urinary content in a sample taken on the next morning. The objective in these studies was to keep the monkey in an optimal clinical condition. In the human clinical situation, this point can be studied much more readily, intravenous tubes would make mineral supplementation much easier. Continuous administration instead of large amounts several times a day would then be possible. Faeces and urine can also be collected separately, so intake and excretion can be accurately determined.

Lethal irradiation severely suppresses the immune system and the bone marrow functions. This is required for successful bone marrow grafting (Van Bekkum and De Vries, 1967b). The outcome of bone marrow transplantation is dependent on many variables, including donor selection (Van Bekkum, 1974a), pretreatment of the graft (Dicke et al., 1972), administration of antilymphocytic serum (Van Bekkum et al., 1972) and success in microflora suppression (Van Bekkum et al., 1977).

Since the grafting experiments were part of other studies, the decontamination series is very dissimilar in this respect and no results on the transplantation studies can be given in this presentation. For the same reason, no results on bone marrow recovery are reported. The biochemical parameters are also not presented for the group of monkeys developing a Graft versus Host reaction, since a GvH reaction affects electrolyte metabolism by lymphocytic infiltration of the intestinal mucosa increasing the electrolyte problems. The liver function may also be affected due to lymphocytic infiltrations (Van Bekkum and De Vries, 1967c).

As mentioned in Chapter II, the husbandry procedure for the monkeys was somewhat changed in early 1974. Instead of cleaning and sterilization with peracetic acid, the cages were replaced every other day by clean ones, which were also sterilized with peracetic acid. The drinking water was provided in a similar bottle as the one used for food instead of in an open trough. Until that time, only one monkey had died before day 20 (at day 18 due to GvH). Beginning in early 1974, it was not possible to maintain the monkeys alive for more than 10-15 days after irradiation. The clinical picture was an acute loss of tonus followed by death within a few hours. The last known biochemical values were well within normal ranges. At necropsy (Dr. Hollander /Dr. Zurcher, Institute for Experimental Gerontology TNO), no clear-cut lesions were found. Acute imbalances in electrolytes were suspected. The monkeys that did survive for more than 20 days showed the same clinical picture but were kept alive by intravenous and subcutaneous administration of large amounts of fluids. More intensive treatment with potassium chloride and administration of fluid in amounts compensating for the daily weight loss had no effect.

The hypothesis that lack of sodium was the cause of death in these monkey is supported by the low sodium levels found in the urine before irradiation (unpublished observations). Increased loss or decreased absorption from the intestinal tract after irradiation resulted in a sodium deficiency. This, however, was not accompanied by a decreasing serum level of sodium and does not explain why the problem became apparent after a minor change in the caretaking procedure. It is also not explained how the other monkeys managed to keep in balance with their sodium intake. Inquiries revealed that the sodium content of the food and drinking water had not changed nor had the sodium content of the disinfecting soap.

The low urinary sodium content observed before irradiation and the clinical improvement observed afterwards led to the decision not to run controls without supplementation to test the hypothesis. There-

fore, any other phenomenon that occurred simultaneously could have attributed to the death of these monkeys: For example, a viral infection inhibiting epithelial regeneration after irradiation was considered. However, no virus could be isolated (Drs. J. Chevour, Dept. of Medical Microbiology, Academic Hospital Leiden, personal communication). Monkeys from the same shipment and therefore exposed to the same environment died without sodium supplementation, but survived when supplemented. However, these monkeys were treated at different times.

A GvH reaction was also improbable, since the monkeys died before the bone marrow showed signs of recovery. Early deaths occurred in monkeys receiving allogeneic grafts, autologous bone marrow or no grafts at all. No changes could be found at the irradiation source which would lead to an increased irradiation dose resulting in an intestinal syndrome.

The decrease in serum protein was probably a result of the catabolic state of the monkey. After irradiation, the intestine does not function well; the result is diminished absorption. All monkeys lose weight after irradiation. This indicates that the body fat and subsequently proteins being metabolized.

In conclusion, total body irradiation with 8.5 Gy in decontaminated monkeys results in a severe diarrhoea which is not necessarily lethal when sufficient levels of electrolytes can be maintained.

CHAPTER VII

ANTIBIOTIC CONCENTRATION DETERMINATION

7.1 INTRODUCTION

In gastrointestinal decontamination, so-called nonabsorbable antibiotics are administered orally for suppression of the endogenous microflora in order to prevent infections from this source. To achieve such suppression, the most suitable antibiotic combination was selected on the basis of a sensitivity test (Chapter II). It is of importance for the understanding of the bacteriological effectiveness of the decontamination to be informed about the actual antibiotic concentration that is achieved in the intestinal lumen. The latter is reflected in the antibiotic concentration in the faeces. Obviously, the faecal concentration should be well above the minimum inhibitory concentration for the intestinal flora as determined in the sensitivity test (Chapter II).

Elimination of the "normal microflora" results in an extreme reduction in the colonization resistance (Van der Waaij et al., 1971b). The antibiotic concentration in the gastrointestinal tract should therefore also be sufficient to prevent colonization by microorganisms that are introduced into the isolator by accident.

In the initial phase of decontamination, rather large oral doses of cephalosporines and aminoglycosides are given in order to rapidly achieve an adequate antibiotic concentration. Of the aminoglycoside antibiotics, neomycin was frequently employed. When administered systemically, this agent is known to be nephrotoxic and ototoxic and may produce neuromuscular blocking (Heilmeyer, 1969a). After oral administration, about 3 % is absorbed and excreted in the urine (Heilmeyer, 1969a). It was therefore investigated whether, in cases of damage to the epithelial lining as occurs as a result of irradiation, the absorbed amounts increase. This in turn may lead to kidney damage and accumulation of the drug as indicated by elevated serum levels.

7.2 MATERIALS AND METHODS

7.2.1 Monkeys

Nine Macaca mulatta (rhesus) monkeys were treated as described in Chapters II and IV. All of the animals discussed here, besides an antifungal agent, initially received the combination of neomycin and cephalothin. As described in Chapter II, the dosage of these antibiotics was 0.5 gram three times per day for 3 days followed by 2 days of 0.5 g of each antibiotic two times a day. The same antibiotics were mixed with the liquid food in a concentration of 1 $mg.ml^{-1}$ and were the only antibiotics administered after the first five days. Before irradiation, the cephalothin (in the food) was replaced by bacitracin. Concentration determinations of the latter were not performed. Data on neomycin for two more monkeys from the treatment period bacitracin-neomycin are also included.

7.2.2 Samples

Faecal material was collected each morning. A weighed portion of about 0.5 g was put into a weighed tube and diluted 1:10 in tryptose phosphate broth (Difco), sealed and stored frozen at -18^{o} C. On thawing, these samples were ready for testing. Blood samples were taken twice a week as long as accessible veins were available. The blood was allowed to clot and centrifuged. The serum was also stored frozen at -18^{o} C.

7.2.3 Determination of the antibiotic activity*

The antibiotic content of the samples was determined by an agar well diffusion assay method. This test is a modification of the method described by Bennett et al. (1966).

Plates of 25 x 25 cm were prepared by filling them with 100 ml presterilized and melted D.S.T. agar (Oxoid). For neomycin testing, they were seeded with an Enterobacter strain sensitive to neomycin (mimimum inhibitory concentration (M.I.C.) 1 $\mu g.ml^{-1}$) but resistant to cephalothin (M.I.C. above 200 $\mu g.ml^{-1}$). For determination of cephalothin,

*The author is very grateful to Mrs. J.M. Davies for performing the antibiotic concentration determinations.

a neomycin resistant (M.I.C. above 100 $\mu g.ml^{-1}$) but cephalosporin sensitive (M.I.C. 1 $\mu g.ml^{-1}$) strain of E.coli was used. The strains were stored in liquid nitrogen in small containers each sufficient to seed one plate.

Holes of 0.9 cm diameter were punched in the plates to form wells for reception of samples and standards. Antibiotic standards were prepared in sterile distilled water and contained 0.01, 0.025, 0.05, 0.1, 0.25 $mg.ml^{-1}$ of the antibiotic to be determined. The holes were filled so that the fluid surface was level with the agar. The samples and standards were investigated in triplicate. Their distribution on the plate was governed by application of a random code working sheet. The plates were incubated overnight at 37° C. The inhibition zones (Fig. 7.1) were then measured with vernier calipers. The average zone sizes

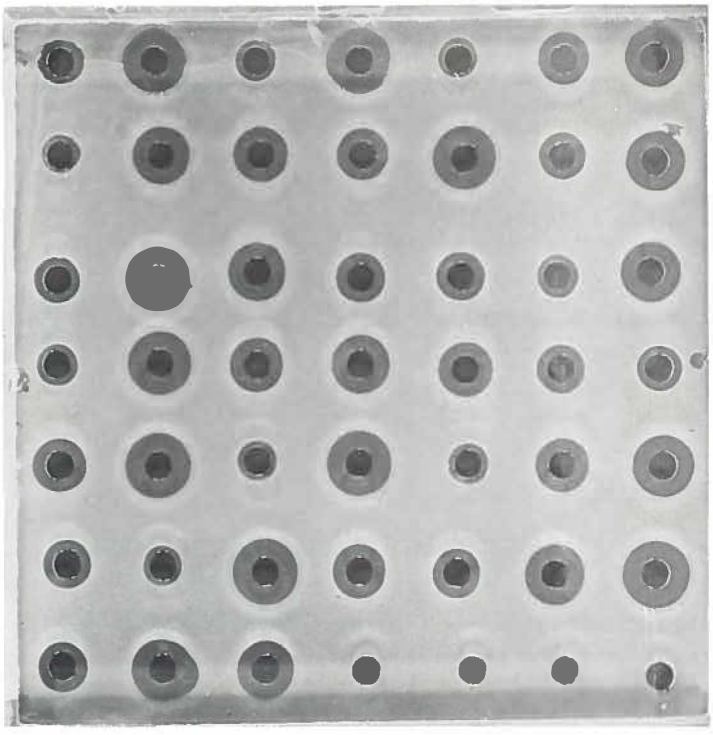

Figure 7.1:

Assay plate showing zones of inhibition

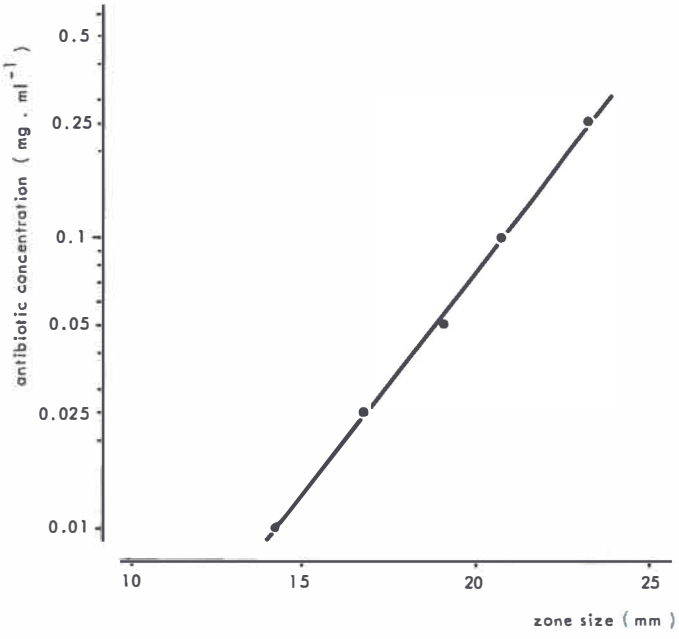

Figure 7.2:
Standard curve for determination of unknown antibiotic concentrations.

were then calculated from both standards and samples. A standard curve was plotted on semilogarithmic paper and the unknown concentration was read from this (Fig. 7.2).

7.2.4 Interpretation of data

The faecal concentration of free active antibiotic substances was related to the antibiotic intake in the previous 24 hours. From day 7 on, the data were pooled per treatment period, these were:

a) from day 7 until irradiation;
b) the first 21 days after irradiation;
c) from day 21 after irradiation until discontinuation of antibiotics or until some solid food (cakes or grains and pellets) was added;
d) the period of addition of solid food until discontinuation of antibiotics.

The amounts of liquid food taken were classified as 1/4, 1/2, 1, 1 1/2, 2 bottles or 0-50, 50-90, 90-180, 180-270, 270-360, etc., ml (Chapter II).

7.3 RESULTS

The mean faecal concentration with standard deviation (SD) in the first 10 days of treatment is plotted for neomycin in Fig. 7.3 and for cephalothin in Fig. 7.4. It is obvious that the faecal concentration was high in the first days of treatment, when 1.5 or 1.0 g of the antibiotic were given, as compared to the days thereafter, when the antibiotics were administered with the food only.

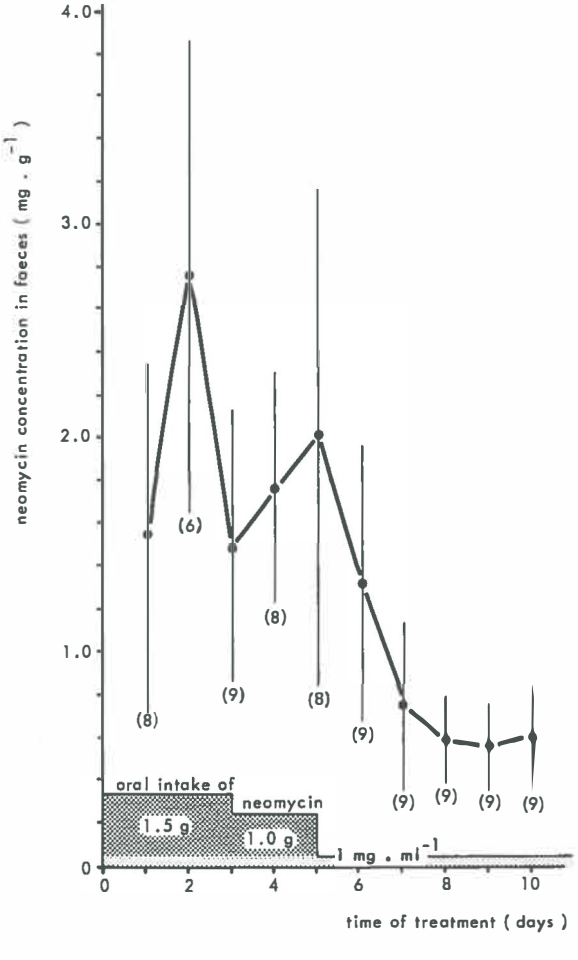

Figure 7.3:
Faecal concentration of neomycin in the first 10 days of treatment.

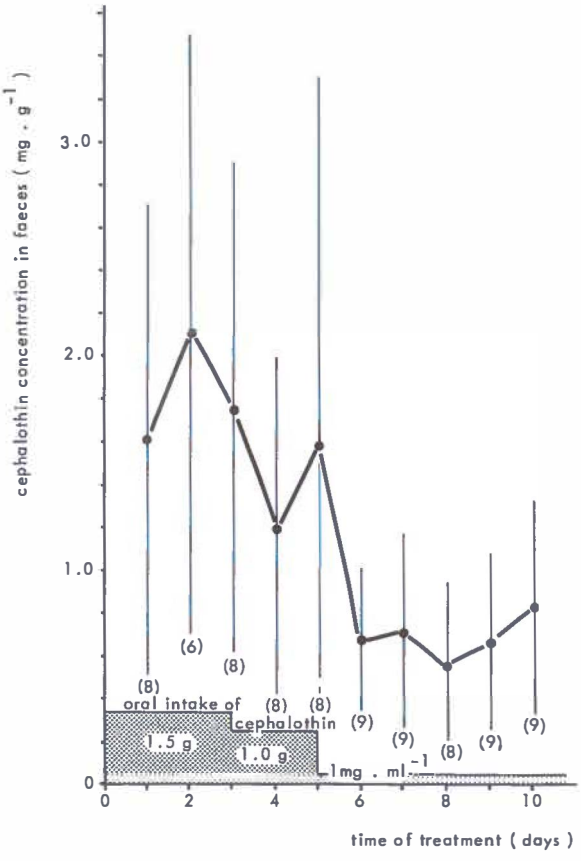

Figure 7.4:

Faecal concentration of cephalothin in the first 10 days of treatment.

The data obtained in the treatment periods mentioned are given for neomycin in Table 7.I and for cephalothin in Table 7.II. For easy comparison, the data obtained for neomycin in the different treatment periods are plotted in Fig. 7.5. The mean faecal concentration of neomycin seems not to be much influenced by the treatment period, unless solid food was also given. The wide range in faecal concentration was due to variation within the data obtained from the individual monkeys.

No or a very low antibiotic activity could be demonstrated in the serum.

Table 7.I

FAECAL CONCENTRATION OF NEOMYCIN IN THE VARIOUS TREATMENT PERIODS RELATED TO THE ORAL INTAKE

	Day 7-irradiation			After irradiation day 0-21			Day 21-solid food			Solid food until discontinuation of oral antibiotics		
Intake in ml of food *	Number of determinations	Mean $mg.g^{-1}$ faeces	SD	Number of determinations	Mean $mg.g^{-1}$ faeces	SD	Number of determinations	Mean $mg.g^{-1}$ faeces	SD	Number of determinations	Mean $mg.g^{-1}$ faeces	SD
0- 50	2	0.39	0.42	6	0.38	0.24						
50- 90				2	0.44	0.57	1	0.50				
90-180	4	0.49	0.01	9	0.44	0.18						
180-270	20	0.57	0.44	22	0.66	0.36	29	0.53	0.29	3	0.19	0.89
270-360	38	0.57	0.18	122	0.57	0.25	103	0.48	0.29	21	0.23	0.17
360-450	78	0.65	0.26	4	0.57	0.34	13	0.71	0.36			
450-540	8	0.53	0.72				3	1.03	0.58			
> 540	28	0.71	0.38				1	1.00				

*containing 1 $mg.ml^{-1}$ of neomycin

Table 7.II

FAECAL CONCENTRATION OF CEPHALOTHIN (after day 7)
RELATED TO THE ORAL INTAKE

Intake in ml of food *	Number of determinations	Mean $mg.g^{-1}$ faeces	SD
0- 50	2	2.75	2.33
50- 90			
90-180	1	0.32	
180-270	8	1.02	0.80
270-360	20	0.54	0.29
360-450	13	0.61	0.51
450-540	2	1.63	0.25
⩾ 540	9	0.62	0.32

*containing 1 $mg.ml^{-1}$ of cephalothin

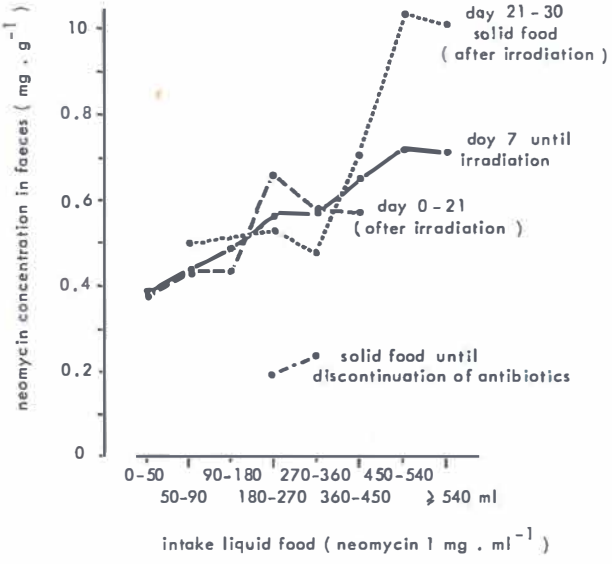

Figure 7.5:

Mean faecal concentration of neomycin after varying oral intake in 4 treatment periods.

7.4 DISCUSSION

After 24 hours of oral antibiotic treatment, the resulting faecal concentrations were found to be sufficiently high for microflora suppression. The faecal concentration of neomycin and cephalothin was well above the overall M.I.C. of 0.19 mg.g^{-1} faeces as indicated by the sensitivity test (Chapter II). According to our results, an antibiotic concentration of 2.1 mg.g^{-1} faeces - the next higher standard antibiotic concentration used in the sensitivity test - was not always achieved. This confirms therefore the rule of thumb that, in this sensitivity test, growth in addition to the four right-hand-bottom cups indicates that the antibiotic combination tested is unsuitable for obtaining a complete suppression of the microflora.

The faecal antibiotic concentration data also indicate a rapid transit time in the intestine, since a noticeable faecal concentration was achieved within 24 h. Within 48 h after discontinuation of the "high dose regimen", a rapid decrease in the antibiotic concentration in the faeces was also observed. This agrees with the (unpublished) observation that, after discontinuation of the oral administration of antibiotics, the faecal concentration decreases rapidly. After 48 hours the amounts of antibiotics found in the faeces were almost zero, allowing recontamination (Chapter VIII).

A similar rapid change in faecal concentration was observed by Van der Waaij et al. (1974b) after oral treatment with gentamicin in a 12-year-old boy. In mice, however, a more gradual increase over several days followed by a similar gradual decrease after discontinuation of the antibiotics was observed (Van der Waaij et al., 1974 b; c). For a better understanding, it is important to know that in decontaminated rodents, in contrast to primates, the caecum enlarges to an extent comparable to what occurs in germfree rodents (Van der Waaij, 1969; Gustafsson and Norin, 1977).

Even when added in small amounts, solid food seems to reduce the amount of biologically available antibiotics in the faeces. It is known that faecal material can inactivate antibiotics in vitro (Wagman et al., 1974). When mixed with dog faeces, neomycin was found to be inactivated by faeces to a great extent. The amount of inactivated antibiotic "appeared to depend on the amount of antibiotics available per gram of faeces" (Wagman et al., 1974). This antibiotic inactivation by intestinal contents could also be the explanation for the wide fluctuations found in the present studies. Despite a constant diet, the faecal composition can vary, for example, in its content of bile salts and cell debris.

Since the amount of biologically available antibiotic seemed to be important in microflora suppression, the determinations were performed with sterile water as controls; therefore, only the available amounts of free antibiotic were determined. The 1:10 dilution in broth might have reactivated the antibiotics to some extent, leading to a somewhat overestimation of the faecal content.

It appears that the inactivation is very variable. The wide range in faecal concentration obtained after intake of a constant amount of antibiotics in a fixed amount of food suggests that there is no point in adjusting the antibiotic content of the food to the daily intake of food in an individual monkey in order to obtain a predetermined faecal antibiotic concentration.

In the period after the high dose regimen, the faecal antibiotic concentration decreased, but remained well above 0.19 $mg.g^{-1}$ faeces. A limited number of unpublished observations, however, demonstrated that microflora suppression could not be achieved when the high dose regimen was omitted.

It is reassuring that, even in the first 21 days after irradiation, when the diarrhoea was extremely severe, the faecal concentration of neomycin remained well within a range in which sufficient protection might be expected.

The absence of significant serum levels of neomycin was described for humans treated orally in periods of hepatic failure. In patients treated orally with neomycin for this condition, only in oliguria were serum levels of neomycin detected (Last and Sherlock, 1960). It was mentioned that damage to the gastrointestinal mucosa might permit systemic absorption but the authors believed that this was not likely to be an important factor (Last and Sherlock, 1960). In mice, a serum level of between 5 and 9 $\mu g.ml^{-1}$ was observed between day 7 and day 12 after a dose of 1440 $mg.kg^{-1}.day^{-1}$. With lower dosages, the serum serum levels were less than 3 $\mu g.ml^{-1}$ (Van der Waaij et al., 1974b). In their initial experiments in monkeys, Van der Waaij et al. (1970) found serum values of less than 10 $\mu g.ml^{-1}$; most of the times, the serum level was in the order of 3 $\mu g.ml^{-1}$ of serum. In the previously published series (Hendriks et al., 1975), much higher serum concentrations were found. These determinations, however, were performed with a strain sensitive to both neomycin and cephalosporin . Besides this, in later experiments, this strain was found to be inhibited by some pretreatment sera in a few monkeys (unpublished observations).

It is reassuring that, in the present series of monkeys, only trace amounts of neomycin were found after irradiation when the epithelial lining was damaged. Limited, also published, observations in-

dicated that, even during Graft versus Host disease, no significant serum antibiotic concentrations were present. Since some neomycin is absorbed and since it is potentially toxic, it is prudent not to administer this agent to individuals with severely reduced kidney function.

Similar observations were made in patients. Preisler et al. (1970) found, even in severe radiation enteritis, serum levels of gentamicin of 0.53 $\mu g.ml^{-1}$. The other positive sera contained less than 0.2 $\mu g.ml^{-1}$. Levi et al. (1973) reported that trace amounts of gentamicin could be detected in 21 sera obtained from 9 patients on 8 occasions. In all instances, the level was less than 0.2 $\mu g.ml^{-1}$.

Antibiotic concentrations of neomycin and cephalothin of more than 200 $\mu g.ml^{-1}$ seem sufficient to suppress the "normal" intestinal flora. This correlates well with the observation that, when the oral cavity was colonized (where for most of the time the local antibiotic concentration must have been low), positive faecal cultures were not automatically obtained as long as the antibiotics were given (Chapters II, IV and VIII). When the faecal cultures did become positive, however, growth could be suppressed easily by a change in antibiotics applied. In contrast, the oral cavity remained often colonized, indicating that, in the oropharynx, another condition is resposible for positive cultures (Chapters II and IV).

These considerations lead to the question of whether it is worthwhile to perform the sensitivity test described in Chapter II at all. In healthy monkeys, the sensitivity test seems unnecessary. Because there has been no preceeding antibiotic treatment, resistant strains are either absent or present in low concentrations and will therefore not become evident in the sensitivity test.

In a hospital setting where patients with an impaired immune system are treated, the situation may be different. These patients often received antibiotics and therefore resistant strains may be present. When antibiotics are administered, the selective pressure favours the dissemination of plasmids coding for resistance to the antibiotics applied in the coliform flora (Anderson et al., 1973). In this situation, decontamination is certain to fail.

The efficacy of the sensitivity test in a clinical setting is the subject of the second study of the E.O.R.T.C. Gnotobiotic Project Group.

CHAPTER VIII

RECONTAMINATION

8.1 INTRODUCTION

Oral administration of suitable nonabsorbable antibiotics in adequate doses leads to an extreme suppression of the gastrointestinal microflora (Chapter II). The microflora suppression has, besides some side-effects (Chapters III and IV), the beneficial effect of protection from endogenous infections (Body et al., 1971; Levi et al., 1973; Levine et al., 1973; Yates and Holland, 1973; Schimpff et al., 1975; Dietrich et al., 1977). However, since the anaerobic bacteria are suppressed, it also has the deleterious effect of minimising the colonization resistance of the gastrointestinal tract (Van der Waaij et al.,1971b). As described for mice, the anaerobic part of the microflora, prevents colonization by potentially pathogenic microorganisms (Van der Waaij et al., 1971b). In the presence of the anaerobic microflora the number of microorganisms required for oral administration resulting in a prolonged colonization of the gastrointestinal tract was 10^6 colony forming units (CFU) while, in germfree mice, this was less than 10^2 CFU. The colonization resistance is dependent not only on the anaerobic microflora but also on the immune system. Irradiation of the mice before challenge led to prolonged colonization by the indicator strain in an increased faecal concentration (Van der Waaij and Heidt, 1977). In the monkeys, it was observed that microorganisms resistant to the antibiotics applied behaved as in mice deprived of the anaerobes (Chapters II and IV). These resistant microorganisms spread to all sites and were present in high numbers. It was expected that a similar mechanism of colonization resistance might exist in primates.

In decontaminated humans, several authors recolonized the gastrointestinal tract in a controlled way before the isolation was discontinued. Dietrich and Fliedner (1973) applied a stepwise procedure. Initially, two species of Bifidobacterium and two species of Bacillus were administered. Thereafter, E. coli and Staph. albus were given; this was followed two weeks later by a faecal specimen of a healthy donor. Raibaud et al. (1975) recontaminated caesarian derived babies by feeding faecal contents obtained from germfree mice that had previously been contaminated with a 10^6 diluted faecal specimen of a

healthy human donor. This procedure resulted in 10^9 CFU of E. coli per gram of faeces. Van der Waaij and co-workers selected a human faecal flora free of potentially pathogenic microorganisms in analogy to the "colonization resistance" flora obtained in mice (Van der Waaij and Heidt, 1977). The microflora selected was obtained from a healthy human volunteer. In irradiated mice, this microflora was found to contain no invasive bacteria. It was also found to be free of yeasts and parasites. No pathogenic viruses could be detected. This microflora was administered to babies after gastrointestinal decontamination (Vossen and van der Waaij, 1973b). The same anaerobic human microflora was administered to the monkeys after the decontamination period in an attempt to restore the colonization resistance before the isolation was discontinued.

Before the anaerobic microflora could colonize the gastrointestinal tract, the antibiotic supply had to be discontinued. This inevitably led to an outgrowth in the gastrointestinal tract of microorganisms that were present; being no longer suppressed by the antibiotics and not yet antagonized by the anaerobic microflora, these persisting microorganisms could colonize the monkey. In this chapter, the effects of the anaerobic microflora itself as well as the chances for abnormal and therefore potentially dangerous colonizations after discontinuation of the antibiotics will be discussed.

8.2 MATERIALS AND METHODS

8.2.1 Monkeys

Of the animals treated as described in Chapters II and IV, 15 rhesus monkeys (Macaca mulatta) survived for prolonged periods in which bone marrow was repopulated. None of the monkeys was a chimaera.

Antibiotic administration was stopped 22-106 days after irradiation (mean, 58.6; median, 52 days). The antibiotics were discontinued only if the leukocyte count had been above $1 \times 10^9 . l^{-1}$, the reticulocyte count above 1 % and the thrombocyte count above $100 \times 10^9 . l^{-1}$ for at least three days.

8.2.2 Food, drinking water and isolation procedures

As described in Chapter II.

8.2.3 Donor microflora

The anaerobic microflora was maintained and propagated in ex-germfree mice housed in isolators such as used in the propagation of germfree animals (Trexler, 1960). Germfree mice were transferred from their isolator into the isolator containing mice associated with the "donor" flora. Germfree techniques were applied. The germfree mice were lodged in cages together with mice already associated with the microflora. When the microflora was to be collected, a number of mice were killed inside the isolator by cervical dislocation. The killed mice were taken out of the isolator and brought into the laboratory. After disinfection of the skin with 70% ethanol, the abdomen was opened. The caecum and colon were taken out and transferred into a sterile Petri dish. Their contents were squeezed out and faecal material was collected and placed into a sterile 2 ml vial. This vial was stored in liquid nitrogen until required. The intestinal contents of 5 mice were placed into one vial. All manipulations were performed with sterile instruments and adequate samples were taken to detect aerobically growing contaminants.

When required for administration to the monkeys, the frozen contents of a vial were submerged in Brewer's thioglycolate broth (Difco) and allowed to thaw at room temperature. Immediately after thawing, the tube was externally sterilized with 2% peracetic acid and brought into the monkey's laminar air flow cabinet. The faecal broth suspensions were given to the monkey orally or, if the animal refused to swallow, a stomach tube was used (Chapter II).

The microflora was administered on three subsequent days starting 48 h after discontinuation of the antibiotics. The nystatin, which does not inhibit the bacterial growth, was continued until the microflora had colonized the intestine.

8.2.4 Microbial monitoring

A Gram-stained smear of the faeces was made daily in order to follow the repopulation of the intestinal tract. The presence of many bacteria, among them half-circular Gram-positive microorganisms lying in clusters, was considered as an indication that the microflora had established itself.

Until establishment of the microflora, swabs were taken daily as described in Chapter II. Thereafter, all swabs became positive in B.H.I. broth, but no growth was obtained from aerobically incubated subin-

oculations onto blood agar plates if the monkey was not previously colonized. The faecal concentration of aerobically growing micro-organisms only was followed either by serial dilution or semiquantitative culturing.

For serial dilution, 0.05 gram of faeces was suspended in 0.5 ml B.H.I. broth in 2 ml cups of a plastic tray. Dilutions were made with 0.05 ml dilutors in 12 cups each containing 0.5 ml B.H.I.broth.The tray was then incubated overnight at 37° C in a tightly closed plastic box. Subinoculations were subsequently made with a stamp onto B.H.I.,Endo, aesculin-azide and mannitol-salt agar. For semiquantitative culturing, a loopful faeces was placed onto the first segment of B.H.I.,Endo, aesculin-azide and mannitol-salt agar. Subsequent segments were streaked with an inoculation needle. Growth only in the first segment was scored as 1+, in only the first and second segment as 2+, etc.

As long as the monkeys were in the laminar air flow cabinets, microorganisms of the same species as found in previous treatment periods were considered as identical (Chapter V). Their isolation was indicated as a "reappearance" if they had colonized the monkey before but were suppressed. At a variable inverval after the donor flora had been established, the isolation was discontinued by taking the monkeys out of the laminar air flow cabinets.

8.3 RESULTS

After the donor flora had been established (usually around the third day of administration), the diarrhoea disappeared and the faeces became a sticky amorphous mass. These faeces had no odor and were brown-grey in colour. With respect to the serum electrolytes, the monkeys had already passed the most difficult period in maintaining normal electrolyte levels when the recontamination was performed. In 4 out of 5 monkeys recontaminated before day 40 after irradiation, a decrease in the serum calcium levels was observed. The lowest values obtained were 1.89, 1.86, 2.08 and 2.07 $mMol.l^{-1}$. These values were seen in the first 4 weeks after recontamination. In these four animals, the serum protein was quite low (55 ± 5 $g.l^{-1}$), while that in the other monkeys was normal (well above 60 $g.l^{-1}$). All other biochemical parameters determined were within normal ranges.

The microorganisms present as a known colonization or in a suppressed state rapidly colonized the whole monkey as soon as the antibiotic treatment was discontinued (Tables 8.I and 8.II). Note that the Strept. faecalis appearing at day 10 did not grow out in the faeces

into numbers as large as the microorganisms that were already present before the donor flora was administered.

Microorganisms that were isolated and suppressed before irradiation and not isolated again after irradiation also did not reappear when the antibiotics were discontinued. Out of 22 "contaminations" by microorganisms isolated for the first time after irradiation 3 microorganisms were isolated again when the antibiotics were stopped. The time interval between the last isolation and discontinuation of the antibiotics was 1, 13 and 23 days. The interval for the microorganisms which were not isolated again was 6-63 days.

Out of 4 suppressed colonizations after irradiation in 2 the microorganisms reappeared. The interval between last isolation and discontinuation of the antibiotics was 6 and 7 days. This interval was for the microorganisms which were not isolated again 2 and 16 days in one monkey.

After discontinuing the antibiotic treatment, three "colonizations" by microorganisms not isolated before were found. Therefore, these have to be considered as results of "breaks in isolation". Two "breaks in isolation" occurred at day 3 (both Strept. faecalis) and the third at day 4 (a sporeforming microorganism). All reached a faecal concentration of about 10^{10} CFU per gram of faeces.

Table 8.I

DISSEMINATION OF MICROORGANISMS PRESENT AT DISCONTINUATION OF ORAL ANTIBIOTICS (in 8 out of 15 monkeys)

Microorganism:	Days after irradiation antibiotics were discontinued	Sites involved	Faecal concentration (^{10}log)	Faecal concentration semi-quantitative
Strept. faecalis	100	oral		3+
Gram-positive rod	59	oral	0-3	
Non-fermenting Gram-negative rod	102	all sites		0-1+
"	22	oral, faecal	N.D.*	N.D.
"	51**	perianal	N.D.	N.D.
Pseudomonas	37***	faecal	4-8	
"	50	all sites	6-8	
Yeast	51**	all sites	4	
"	37***	oral	6	
"	31	oral	N.D.	N.D.

* N.D.: not determined
** same monkey
***same monkey

Table 8.II

COLONIZATION PATTERN OF MICROORGANISMS THAT REAPPEARED
(after discontinuation of oral antibiotics)
(in 5 out of 15 monkeys)

Microorganism:	Days after irradiation antibiotics were discontinued	First day of reappearance after discontinuation of antibiotics	Faecal concentration (^{10}log)	Faecal concentration semi-quantitative	Observation period in days
Staph. aureus	22	3	2-6		21E
Strept. faecalis	106	2		3+	25E
" "	38	10	0-1		23†
Enterobacter sp.	25	1	9		25E
Klebsiella sp.	65	2	7-10		7E

† Monkey died.
E End of isolation

8.4 DISCUSSION

After termination of antibiotic treatment, the association of the monkeys with the human donor flora allowed reconventionalization without an undue risk of microbial invasion, despite the fact that the immune capacity was still low early after irradiation (Rádl et al., 1974). Colonization of the monkey by the donor flora did not reduce the faecal concentration of microorganisms that colonized the gut before the donor microflora had been established. A similar pattern has also been observed in healthy monkeys (Hendriks, unpublished observations).

It can be concluded that, in previously irradiated monkeys, the donor flora did not restore the colonization resistance to normal levels. In healthy mice, a species for which this human flora may be less suitable, the flora has the potential of reducing the faecal concentration of microorganisms which are already present before the flora is implanted (Chapter IX). For practical purposes, however, the colonization resistance in the monkeys proved to be sufficient, since it permitted the housing of these still immunologically deficient animals in conventional monkey houses. This is also supported by the observation that the Strept. faecalis that appeared at day 10 after the discontinuation of decontamination, i.e., after the microflora had been established, did not grow out into high numbers. In the conventional monkey house in which the animals were not isolated, the monkeys could obtain a normal monkey flora. That this occurred could be concluded from the fact that they rapidly developed normal faecal pellets.

These observations indicate that the microflora which was human derived and maintained in mice is not optimal for monkeys or is incomplete. Unpublished observations of Van der Waaij indicated that the anaerobic mouse flora gives a lower colonization resistance in rats as compared to mice. This species specificity might also exist in primates.

Another explanation could be that the mouse is not suitable for propagation of all strains of the microflora which are essential in rhesus monkeys. This, however, could not be investigated due to the lack of special equipment required for proper culturing of the anaerobic flora.

Observations in human leukaemic patients indicated that an impaired immune system also reduces the colonization resistance (Van der Waaij et al., 1972c). In the previously irradiated monkeys in our study, incomplete immunological recovery at the time of reconventiona-

lization might also be a factor. Preliminary data indicated that, after decontamination followed by recontamination with the donor flora, 10^7 CFU of *E. coli* were required in order to obtain a faecal concentration of 10^8 CFU.g^{-1} faeces for 14 days in unirradiated monkeys. In irradiated monkeys, 10^3 CFU of *E. coli* were sufficient to obtain this faecal concentration when administered at day 52 and day 109 after irradiation.

The interval between termination of antibiotic treatment and establishment of the donor microflora seems to be the most hazardous period. Microorganisms which were only suppressed during the decontamination period are no longer inhibited by antibiotics and not yet antagonized by the anaerobic donor microflora. No "reappearance" from the treatment period of before irradiation was found. The immediate post-irradiation period functioned as a selection mechanism: if microorganisms were not eliminated before irradiation but only suppressed, they reappeared already shortly after irradiation.

When bacteria were present at the time of termination of antibiotic treatment these microorganisms would grow out into high numbers and might become invasive.

It will be discussed in Chapter IX which antimicrobial agents can be administered systemically to treat infections without damaging the donor flora and thereby reducing the colonization resistance.

Recontamination with the human donor flora was found to be a safe procedure, since a) the microflora restored the colonization resistance to such an extent that the reconventionalization was possible; b) the procedure is a rapid one, the interval of between being unprotected by antibiotics and not yet protected by a microflora was short; and c) no potentially pathogenic microorganisms were deliberately introduced as was done in other procedures (Dietrich and Fliedner, 1973; Raibaud et al., 1975).

CHAPTER IX

SELECTION OF ANTIBIOTICS SUITABLE FOR SYSTEMIC ADMINISTRATION DURING RECONTAMINATION

9.1 INTRODUCTION

When the chance for infections from endogenous sources is no longer increased due to a sufficiently restored defense capacity in a decontaminated individual, the oral antibiotics should be discontinued. As discussed in Chapter VIII, termination of the antibiotic treatment leaves the individual unprotected from microbial colonization. Any microorganism present due to persistance or to a break in isolation in this critical phase will multiply and, not being antagonized by the normal microflora, will colonize in the gastrointestinal tract. A high density of a particular microorganism in the intestines can result in invasion by that microorganism (Van der Waaij et al., 1978). These invading bacteria can cause septicaemia, so that systemic antibiotic therapy becomes necessary.

Also when, despite antibiotic decontamination of the digestive tract, an individual suffers from a bacterial infection, systemic antibiotic treatment is required. Despite this systemic antibiotic treatment, the oral antibiotics should be discontinued and recontamination should be performed as soon as the defense capacity has been sufficiently restored.

As discussed in Chapter VIII, a human donor flora was administered in the monkey model. This microflora restored the colonization resistance to a certain extent. Systemically administered antibiotics can have a negative effect on the colonization resistance of normal mice (Van der Waaij et al.,1972b). Application of these drugs by this route therefore jeopardizes the recontamination procedure when they kill or prevent the normal outgrowth of essential strains of the donor flora.

In this chapter, the screening of several antimicrobial agents currently in use in systemic therapy is discussed with regard to their effect on the colonization resistance exhibited by the human donor flora. Since insufficient numbers of monkeys were available for this study, these experiments were performed with germfree mice.

9.2 MATERIALS AND METHODS

9.2.1 Mice

One antibiotic was tested per experiment. For each experiment, 24 germfree random bred ND_2 mice of the same sex, ± 25 g body weight, aged 12 ± 2 weeks were used. They were put under aseptic conditions in a 26x36x15 cm presterilized polycarbonate cage and housed in a laminar air flow bench sterilized with 2% peracetic acid (Van der Waaij and Andreas,1971c). Food, bedding and drinking water were also sterilized. The same precautions as described in Chapter II for handling the monkeys were taken into account with the mice. An overnight culture in B.H.I. broth (Oxoid) of an "indicator strain" of Enterobacter (code number 1881) was added to the drinking water.

To verify the colonization of all mice, a faecal pellet was obtained from each animal after 3 days. The mice were then put into separate autoclaved polycarbonate cages, provided with wire mesh instead of bedding, for further treatment. The 24 separately caged mice were divided into three groups of 8 mice and placed into different compartments of a laminar air flow bench. Care was taken to prevent cross contamination among the three groups of mice during treatment (Van der Waaij and Andreas, 1971c).

9.2.2 Treatment groups

Group I : received the antibiotic to be tested intraperitoneally daily for 12 days starting at day zero. The antibiotic was dissolved in 0.1 ml of distilled water.

Group II : received the antibiotic in a similar way; in addition, they were given the human donor flora at 24, 48 and 72 hours after starting the antibiotic. The donor flora was prepared as described in Chapter VIII. Each mouse received 0.1 ml of the suspension orally each time.

Group III: was only orally treated with the donor flora as described above (II), but did not receive the antibiotic.

9.2.3 Microbiological monitoring

Faecal samples were obtained from each mouse before or on the day that the antibiotic was started and thereafter every other day. A

faecal pellet (± 0.05 g) was suspended in 0.5 ml B.H.I. broth and diluted serially 1:11 in a tray of 8x8 wells of 2 ml each. Dilutions were made with dilutor loops of 0.05 ml which were heat sterilized and cooled before each dilution step.

The tray was put into a plastic box which was closed tightly to prevent dehydration. After overnight incubation at 37° C, subinoculations were made with a stamp onto Endo agar (Difco). After an additional overnight incubation at 37° C, the Endo agar plates were inspected for growth. The faecal concentration could then be read and was expressed as the ^{10}log of the number of colony forming units (CFU) of the "indicator strain". For each treatment group, the mean of the ^{10}log of the faecal concentration of 8 mice is plotted in the figures.

9.2.4 Antibiotics tested

Penicillin G	(Mycofarm)	10,000	IU	divided	into	2	doses	(4.10^6 $IU.kg^{-1}$ per day)
Ampicillin	(Amfipen)	2	mg	"	"	2	"	(80 $mg.kg^{-1}$ per day)
Cephalothin	(Keflin)	2	mg	"	"	2	"	(80 $mg.kg^{-1}$ per day)
Carbenicillin	(Pyopen)	10	mg	"	"	2	"	(400 $mg.kg^{-1}$ per day)
Oxytetracycline	(Terramycin)	1.3	mg	"	"	2	"	(52 $mg.kg^{-1}$ per day)
Doxycycline	(Vibramycin)	0.1	mg	"	"	2	"	(4 $mg.kg^{-1}$ per day)
Erythromycin	(Erythrocine)	0.35	mg	"	"	2	"	(14 $mg.kg^{-1}$ per day)
Clindamycin	(Dalacin C)	1	mg	"	"	2	"	(40 $mg.kg^{-1}$ per day)
Gentamicin	(Garamycin)	0.25	mg	"	"	2	"	(5 $mg.kg^{-1}$ per day)
Gentamicin + Cephalothin	as above.							

9.3 RESULTS

The mean faecal concentration of the "indicator (Enterobacter) strain" is plotted in the figures for each treatment group for each antibiotic tested. The "indicator strain" reached a faecal concentration, of at least 10^8 CFU per faecal pellet. Systemic antibiotic treatment in the absence of an anaerobic gastrointestinal microflora did not affect the faecal concentration of the "indicator strain", irrespective of whether the strain was sensitive or not to the antibiotics applied. The only exception was seen during carbenicillin treatment, when a transient decrease in the faecal concentration of the indicator strain was observed.

The human donor flora decreased the faecal concentration of the "indicator strain" to about 10^4 CFU/faecal pellet. In mice treated with carbenicillin, erythromycin or clindamycin (Figs. 9.4, 9.7 and

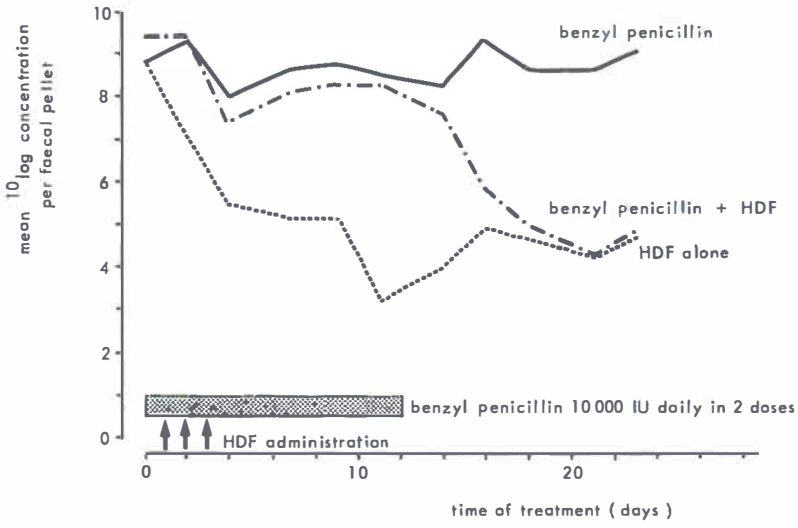

Figure 9.1:

Effect of systemic administration of benzyl penicillin on the human donor flora.

9.8), the "indicator strain" remained present in a concentration well above that of the mice which received only the donor flora.

In mice treated with benzyl penicillin, ampicillin (Figs. 9.1, 9.2 or the combination of gentamicin and cephalothin (Fig. 9.10) (and to some extent when treated with doxycycline) (Fig. 9.6), the faecal con-

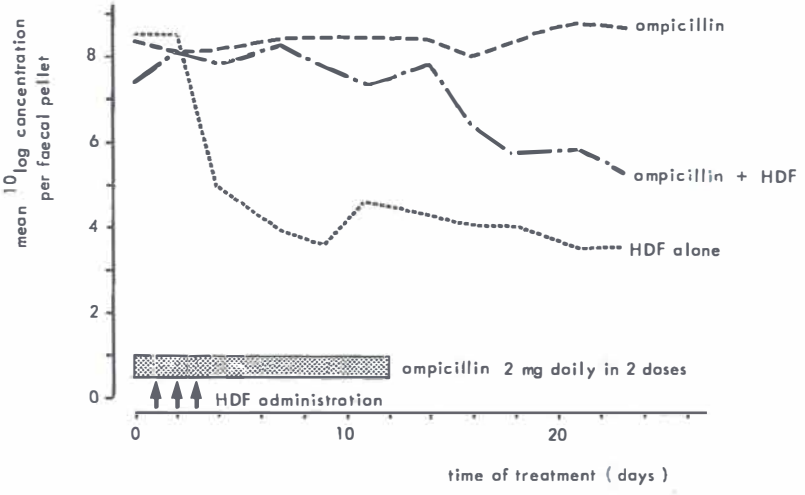

Figure 9.2:

Effect of systemic administration of ampicillin on the human donor flora.

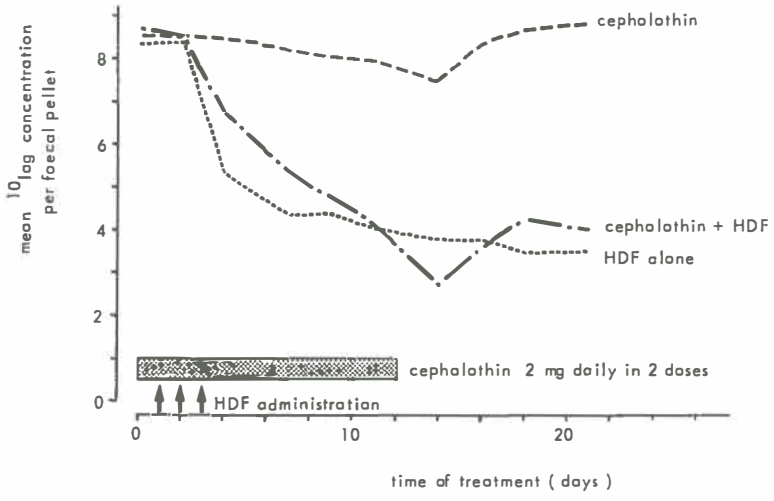

Figure 9.3:

Effect of systemic administration of cephalothin on the human donor flora.

centration remained high as long as the antibiotic was administered, but decreased after discontinuation of the drug, indicating that essential parts of the donor flora were only inhibited. In mice treated with cephalothin, oxytetracycline or gentamicin (Figs. 9.3, 9.5 and 9.9), the faecal concentration was reduced to levels comparable to those achieved when only the donor flora was administered, indicating that the donor flora was not affected.

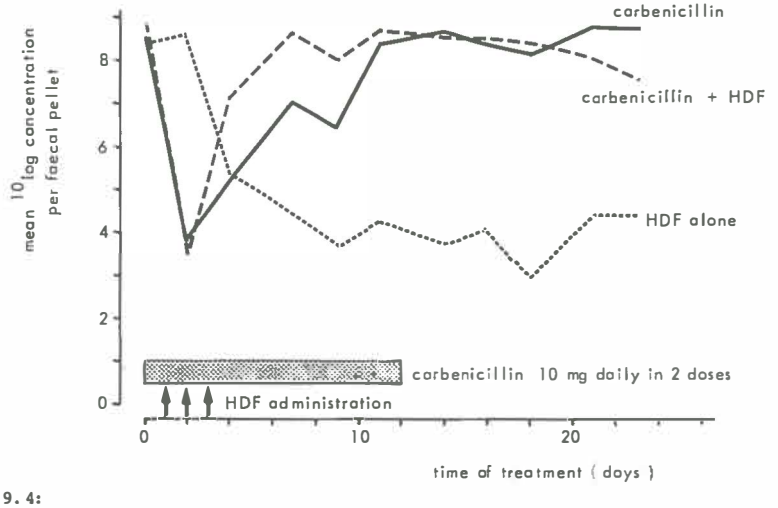

Figure 9.4:

Effect of systemic administration of carbenicillin on the human donor flora.

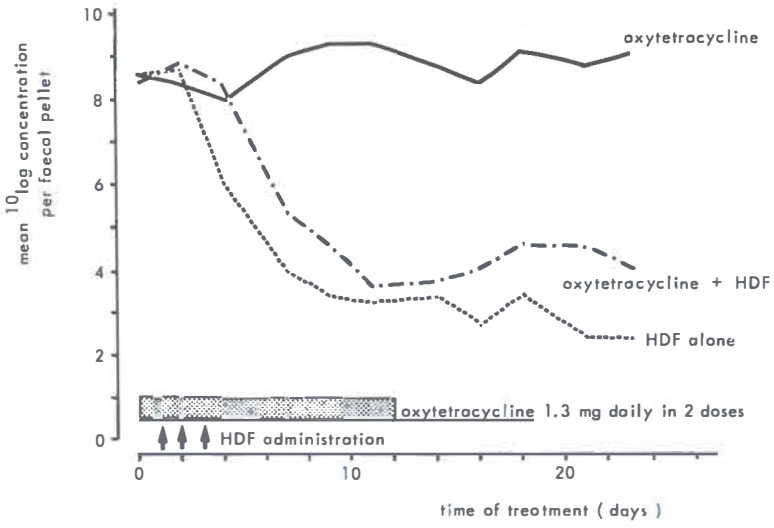

Figure 9.5:

Effect of systemic administration of oxytetracycline on the human donor flora.

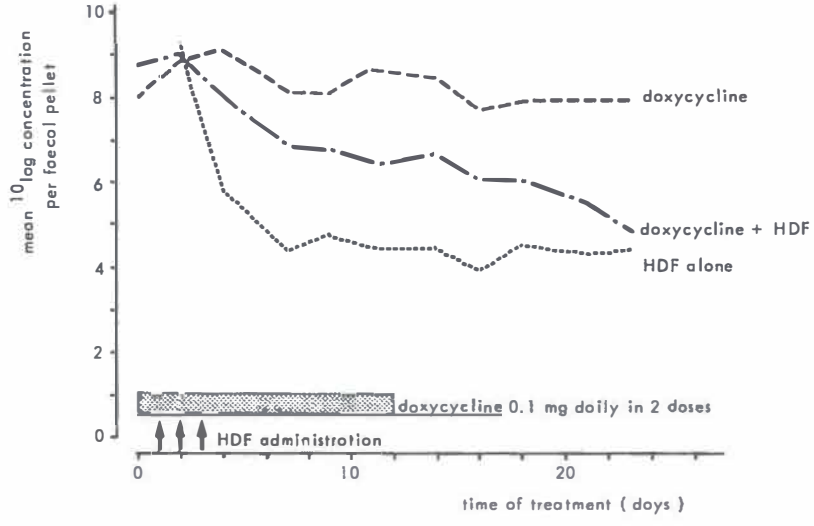

Figure 9.6:

Effect of systemic administration of doxycycline on the human donor flora.

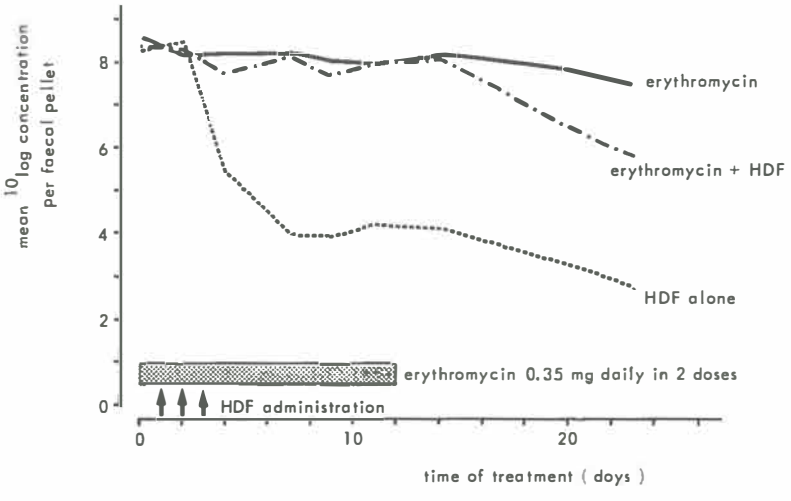

Figure 9.7:

Effect of systemic administration of erythromycin on the human donor flora.

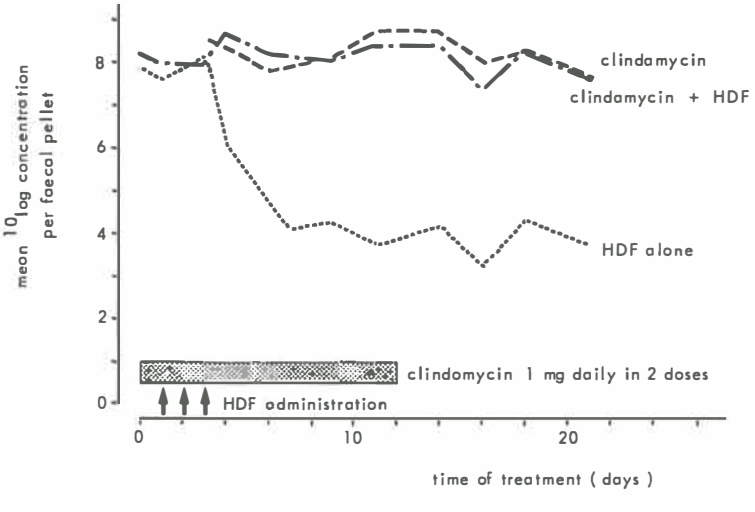

Figure 9.8:

Effect of systemic administration of clindamycin on the human donor flora.

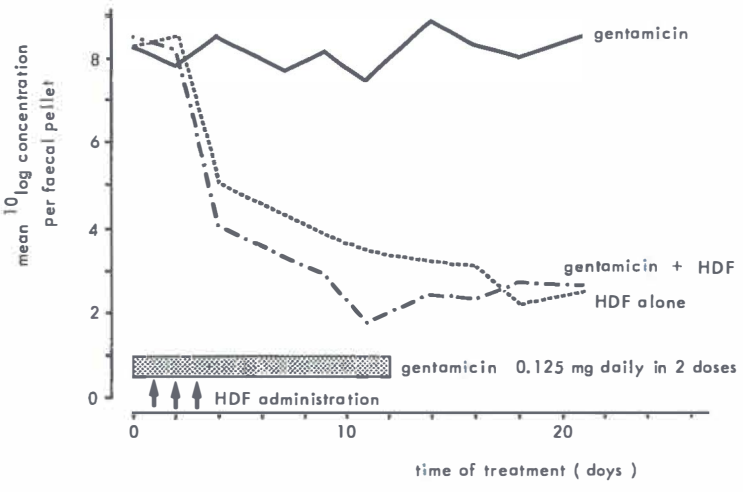

Figure 9.9:

Effect of systemic administration of gentamicin on the human donor flora.

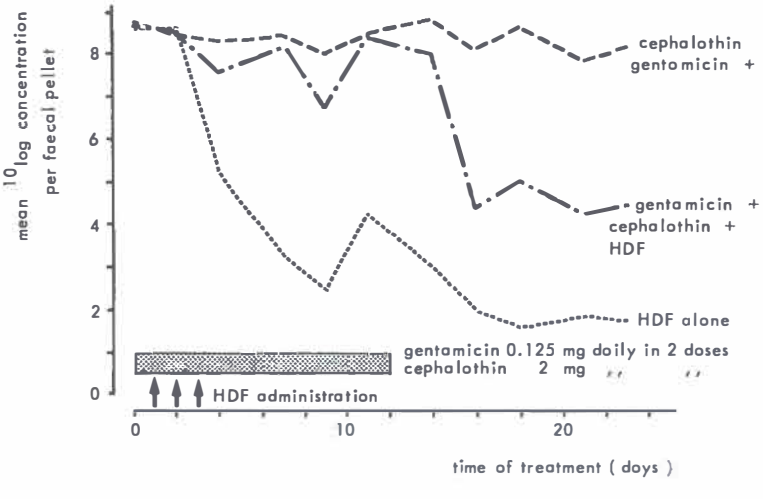

Figure 9.10:

Effect of systemic administration of cephalothin and gentamicin on the human donor flora.

9.4 DISCUSSION

When oral antibiotics can be discontinued in gastrointestinal decontamination, the normal microflora has to be restored in order to restore the colonization resistance. If, however, systemic antibiotic treatment is required, the drugs given should not interfere with outgrowth of the donor flora. Obviously, agents like clindamycin, erythromycin and carbenicillin which are, among others, clinically in use for treatment of anaerobic infections should be avoided. Also those agents such as benzyl penicillin, ampicillin, doxycycline and the combination of cephalothin and gentamicin that do not kill but do prevent the normal outgrowth of the donor flora should be avoided.

The results obtained are in agreement with the observation that the anaerobic flora controls the aerobic part of the microflora (Van der Waaij et al., 1971b). In the absence of human donor flora, the "indicator strain" was present at 10^{8}-10^{9} CFU.g^{-1} faeces. When the anaerobes were administered, the concentration was decreased by about 4 10^{10} log.

It should be realized that the mouse is not the normal host for this human derived microflora and therefore the colonization resistance obtained in mice is not as high as in humans. For instance, in a 26-year-old human female, decontamination failed to eradicate a Candida albicans. This microorganism could not be suppressed with amphotericin B. It was present in a concentration of 10^{11} CFU.g^{-1} faeces. After discontinuation of the oral antibiotics and administration of the donor flora, the faecal concentration decreased sharply to less than 10^{2} CFU.g^{-1} faeces (Van der Waaij and Vossen, 1973b,c).

The model determines the effect on the colonization resistance when recontamination is performed in the presence of antibiotics. It is uncertain, however, whether the results can be extrapolated to the conventional clinical situation when a normal flora is present and antimicrobial agents are administered. In this situation, the colonization resistance in mice was also affected by the administered antibiotics (Van der Waaij et al., 1972b; Thym and Van der Waaij, 1979).

As mentioned in Chapter VIII, it was not attempted to separate the donor flora into single strains, which when mixed should restore the colonization resistance. It was impossible therefore to determine the sensitivity of the individual strains to antimicrobial agents.

Within the group of aminoglycosides, the spectrum of activity is rather constant and does not include anaerobes. Gentamicin is also not excreted in the bile in significant amounts (Heilmeyer, 1969b). It is probable therefore that gentamicin can be administered without af-

fecting the anaerobic microflora. With regard to penicillin, ampicillin and carbenicillin, it is probably better to avoid these during recontamination, since they are excreted to some extent in the bile (Heilmeyer, 1969c). Since all penicillin-like substances have a comparable pharmacology and all have an effect on anaerobes (Sutter and Finegold, 1976), it is probably best to avoid all of them during recontamination.

The cephalosporins, however, show a quite different range of activity within the group. Cephalothin was found not to exhibit an effect on the colonization resistance in the test described. The various cephalosporins should be individually tested before it is decided whether they can be administered during recontamination.

Tetracyclines are known to affect the intestinal microflora; when administered orally, 20-50 % of the oral dose is found in the faeces (Heilmeyer, 1969d). When administered systemically, the amount in the faeces is 6-10 % of the dose. It is a matter of speculation whether the other "old" tetracyclines behave like oxytetracycline. The newer doxycycline exerted an inhibitory effect on the donor flora. Because doxycycline is excreted rather slowly (Heilmeyer, 1969e), it is obvious that its effect continues beyond the treatment period. It is uncertain whether the data for doxycycline can be extrapolated to minocycline. These data are not very important for practical application, since these agents are generally not used in immunologically debilitated patients due to their bacteriostatic mode of action (Garrod et al., 1973).

Rather astonishing is the observation that gentamicin and cephalothin, which when given separately do not affect the donor flora, have a definite effect when administered together. Minimal amounts of gentamicin probably also appear in the faeces and, due to their synergistic action, affect the colonization resistance.

CHAPTER X

GENERAL DISCUSSION

Aspects of gnotobiotic treatment of rhesus monkeys are described in this work. The study was performed in order to obtain a preclinical model for bone marrow transplantation which is more closely related to the human situation than are mice.

The main aspects of the investigation were:

1) How to produce monkeys free of microorganisms and what are the side effects of this treatment;
2) How to keep these monkeys free of microorganisms after irradiation, what is the meaning of a positive culture and what are the side effects in this treatment interval;
3) How to discontinue the gnotobiotic treatment.

After oral administration of nonabsorbable antibiotics, the microflora of healthy monkeys could be rapidly suppressed. If the flora was not suppressed at day 4, however, chances of obtaining a complete microflora suppression were very poor (Chapter II). Reduction of the antibiotic intake resulted in the appearance of colonizations; but, these could be suppressed by adjustment of the antibiotic regimen. After seven days of treatment, a number of "new colonizations" were found. These were attributed to "breaks in isolation", but since they appeared more frequently in the first weeks of treatment as compared to later treatment periods, it was concluded that a number of these "breaks in isolation" were in fact escapes from antibiotic suppression. At the end of the preirradiation treatment interval one half of the monkeys showed a complete suppression of the microflora. The oral cavity was the side remaining most frequently colonized (Chapter II).

The faeces were found to contain sufficient amounts of antibiotics to suppress all microorganisms (Chapter VII).

Despite several alterations in metabolism, the monkeys thrived well and showed no clinical side effects. Besides a decrease in the haematocrit, no changes in the parameters monitored were observed (Chapter III).

The conclusion from this part of the study therefore is that gastrointestinal decontamination is quite feasible in healthy monkeys.

After irradiation and bone marrow grafting, there is an interval of immunodeficiency. The length of the interval and the severity of the impairment of the immune system depends on the nature of the bone marrow graft. The main objective of the study, elimination of endogenous microflora, should find expression in this treatment period; however, colonizations were encountered in this period. They originated from 3 sources: 1) those present at irradiation. The Gram-negatives of these microorganisms present at irradiation frequently colonized the other sampled sites after irradiation; 2) "reappearance" of microorganisms that colonized the monkey, but were suppressed before irradiation; 3) "break in isolation". As in the preirradiation interval, the chance for isolation of these microorganisms was quite different in the several 10-day periods considered. It was discussed that a number of these "breaks" were escapes from suppression and therefore also "reappearances" (Chapter IV).

When cultures unexpectedly showed growth after being considered negative, the chance for a new colonization was roughly 50%. The other isolates were a contamination. Contaminations found before irradiation could reappear after irradiation as a contamination or as a colonization. Colonizations found and suppressed before irradiation could also reappear after irradiation as contaminations and as colonizations.

After irradiation, the diarrhoea increased and led to severe loss of electrolytes. Initially, supplementation with potassium was sufficient to keep the monkeys alive; later, sodium was also required (Chapter VI).

Although the intake of antibiotic containing food decreased after irradiation, the antibiotic level of the faeces remained well above 0.2 $mg.ml^{-1}$. After solid food was added, the faecal antibiotic concentration decreased (Chapter VII).

The conclusion to be drawn from this treatment interval is that decontamination is quite feasible after irradiation but requires careful supplementation with electrolytes, particularly sodium and potassium. It is also possible to control the microflora in this interval, although several colonizations have to be dealt with. The most important conclusion from this study is that absolute eradication of microorganisms can not be guaranteed.

The microorganisms that colonized the monkeys were frequently found in the oral cavity. In this area, the contact time with the antibiotics was very short and there are many niches where the microorganisms can be shielded from the antibiotics. It is probably also from these niches that microorganisms "reappear" or "escape" the suppression.

In several studies on patients with severe immunosuppression due to the basic disease "nonlymphocytic leukaemia" and its treatment, it was reported to be difficult to decontaminate the oral cavity (Levine et al., 1973; Schimpff et al., 1975; Levitan and Perry, 1967).

In an attempt to prolong the activity of the antibiotics in the oral cavity of patients, 2% amphotericin B was mixed with Orabase®. Orabase is a paste which adheres to the mucosa and gradually releases its premixed drug content. This provides a presence of the antibiotic for several hours in the saliva (de Vries-Hospers and Van der Waaij, 1978).

Another approach would be to attempt to eradicate the foci where microorganisms survive by systemic antibiotic treatment. An attempt was made by Enno et al. (1978), who added cotrimoxazole to their regimen of nonabsorbable antibiotics (framycetin, colistin and nystatin). The number of infections appeared to decrease as compared to patients treated with nonabsorbable antibiotics alone. Systemic administration of more potent agents such as gentamicin in combination with cephalosporin or carbenicillin might be more effective but seems waranted only in case of an infection. Too many side effects were found when these combinations were administered on a therapeutic basis (Schimpff et al., 1978).

In the monkeys, the microflora of the skin disappeared spontaneously. In man, however, skin disinfection is required in order to suppress the skin flora. The same is required for the genital area.

The isolation efficacy is difficult to evaluate. The microflora present when the isolation period started was not typed, so no comparisons could be made. However, if such typing had been performed, it would remain a matter of speculation whether all microorganisms were present in detectable quantities. It is quite conceivable that only after elimination of the competitive microflora would microorganisms become detectable when they were only present in a niche.

Another factor interfering in the evaluation of the isolation efficacy is the presence of the antibiotics. Microorganisms introduced into the isolator but sensitive to the antibiotics applied remain undetected.

Isolation systems are in fact only a number of precautions taken to minimalize the chance that a microorganism can reach the isolated individual. A conventional type of an isolation room with a multidirectional ventilation system is designed to dilute airborne contamination and to maintain an overpressure to the adjacent rooms. Because the attendants, although dressed in cap and gown when entering the

isolation room, take their normal flora with them, the system cannot be absolute. Furthermore, a ventilation system based on pressure difference between rooms may in practice not function properly (Williams and Harding, 1969). Temperature gradients may override the pressure-difference by transport of the warm air along the upperside of the door frame, the cold air going in the opposite direction along the lower site (Bouwman, 1972).

In our system, it was decided to apply air laminar flow isolators because these are not based on airborne contamination control by over-pressure. Temperature gradients do not occur as long as these cabinets recirculate air inside the room in which they are placed. When the attendants remain downstream of the subject treated and apply aseptic techniques, there is at least a (theoretically) complete reverse isolation.

The most important route (expressed as number of microorganisms) by which microbes can apparently reach and contaminate an isolated individual is the food. For monkeys, grains and pellets can be autoclaved but, in the clinical situation, this is more difficult, as steam sterilization affects the taste of food. In the hospital setting, food sterilization is important because normal hospital food contains bacteria of which, in terms of infections, the Gram-negatives are the most important. This is particularly true when it has been kept warm for prolonged periods, as occurs when food is obtained from a central kitchen. Raw meat has been incriminated as the ultimate source of contamination (Cooke et al., 1970; Shooter et al., 1971). The observation that cooked food became contaminated by means of the bowls and utensils has been used to explain its bacterial load (Shooter et al., 1970). Since contamination by any microorganism should be prevented during isolation, separately prepared sterile food is therefore mandatory in decontamination.

If we assume that the cluster of initial isolations of microorganisms in the first period after irradiation are due to "reappearances" and not to "breaks in isolation" (Chapters IV and V), the laminar air flow isolation system together with the other precautions described functioned very well in our experiments. This could be deduced from the small number of "breaks in isolation" in the periods following the first 10 days after irradiation when the animals appeared to be extremely susceptible to contaminations.

In studies performed on patients treated with bone marrow transplantation or suffering from acute leukaemia, gastrointestinal decontamination has been found to be effective in prevention of infection (Bodey et al., 1968b; 1971; 1979; Levi et al., 1973; Levine et al.,

1973; Yates and Holland, 1973; Dietrich et al., 1977; Buckner et al., 1978). The data presented by these authors indicate an enormous reduction in faecal flora. Decontamination was therefore considered as successful from the clinical point of view. It was not mentioned, however, whether the patient's oropharynx swabs and faeces were free of living (aerobic) microorganisms during the entire treatment period. In the study of the E.O.R.T.C. Gnotobiotic Project Group (Dankert et al., 1978), only 14% of the patients were free of aerobic microorganisms during the entire remission induction period and were therefore considered as successfully decontaminated from the bacteriological point of view. In the present study with monkeys, all failures were presented from the bacteriological point of view and it was attempted to also indicate their clinical relevance.

The third aspect of the study concerns the discontinuation of decontamination. When the immune capacity is restored, the supportive treatment can be discontinued. This creates a hazardous interval analogous to the situation described for a microorganism resistant to the antibiotics. When the antibiotics are discontinued, any microorganism present (as a colonization or in a suppressed state) is no longer antagonized and will grow out into high numbers (Van der Waaij,1971b). To restore the normal situation as soon as possible a human anaerobic donor flora was administered in an attempt to reestablish the colonization resistance. It was found that this could be done in the monkeys without major difficulties, but microorganisms present at the time of administration of the donor flora remained present in a high concentration (Chapter VIII). The monkeys could be taken out of the isolator without undue risk for infection after being associated with this donor flora.

If antibiotic treatment is also required in the period of administration of the donor flora, the effect of the antibiotics on the donor flora should be known. This was studied in a mouse model. The effect of the most often used antibiotics could be divided into: 1) no effect (gentamicin, cephalothin, oxytetracycline); 2) inhibitory effect as long as the agent was present (gentamicin + cephalothin, ampicillin and doxycycline); 3) permanent damage (clindamycin, erythromycin, carbenicillin) (Chapter IX).

The major disadvantage of gastrointestinal decontamination is - from the bacteriological point of view - the total elimination of the intestinal microflora and thereby complete abolishment of the colonization resistance. When an individual is unable to take the oral antibiotics for some reason, he is unprotected from microorganisms present

anywhere inside the isolation system. In leukaemic patients, strictly anaerobic bacteria rarely cause infections, while the Gram-negative bacteria and to some extent the Staphylococcus aureus are the major offending microorganisms (Bodey, 1975). It would be of advantage therefore if the latter group of microorganisms could be eliminated selectively, i.e., without affecting the microorganisms responsible for the colonization resistance. In this situation, an individual would be protected from uncontrolled overgrowth by the endogenous flora even during periods when the antibiotics cannot be taken because of nausea or for any other reasons. The efficacy of selective decontamination in granulocytopenic patients has been demonstrated (De Vries-Hospers et al., 1979). These patients were not submitted to any form of isolation.

The results presented here indicate that absence of microorganisms cannot be guaranteed, even not when all cultures are negative. Microorganisms can appear in cultures of samples of decontaminated individuals because of introduction due to a "break in isolation" or they may have persisted somewhere in the animal. This may have severe consequences with regard to bone marrow transplantation, since the presence of a conventional microflora seems to influence the development and severity of a Graft versus Host reaction following bone marrow transplantation. In germfree and decontaminated mice, it has been found that, after transplantation of incompatible bone marrow, no clinically overt Graft versus Host disease developed (Jones et al., 1971; Van Bekkum et al., 1974; Heit et al., 1977). However, a colonization by a Gram-negative bacteria might enhance a Graft versus Host reaction when occurring in the first period after irradiation (Van Bekkum et al., 1977b). These consequences of failure of decontamination in bone marrow transplantation need further study and are not discussed in this thesis.

Furthermore, the question remains: should this supportive treatment be applied in a clinical situation? In the clinical trials in man, despite a significantly decreased number of infections, no improvement was found with regard to long term survival in leukaemia (Levi et al., 1973; Levine et al., 1973; Yates and Holland, 1973; Dietrich et al., 1977). Exceptions were the studies of Bodey et al. (1968b) and Schimpff et al. (1975), who found an increased survival rate. As long as a more aggressive antileukaemic treatment is not applied in the decontaminated patients, the only gain is fewer infections and apparently not an increased life expectancy. The cost is a

severe psychological stress on the patient and on the attendants (Holland et al., 1967; Kamphuis, 1979; Kohle, 1979). The financial costs are also high (Pizzo and Levine, 1977). In a conventional setting, however, these vulnerable patients are often nursed in a separate room. The psychological stress of conventional isolation is also severe. In a separate room, communication with a patient is more difficult as compared to strict reverse isolation where visitors can come close to the isolator.

With regard to the number of infections in leukaemic remission induction therapy (Bodey, 1975), there seems to be a case for gnotobiotic treatment. However, the cost benefit analysis seems to be negative for total decontamination and strict reverse isolation in a conventional remission induction regimen. The balance might become positive for selective decontamination.

In patients receiving an unfractionated autologous bone marrow graft, the period of immunosuppression is relatively short and no Graft versus Host reaction can develop. Selective decontamination is therefore probably sufficient and total decontamination under conditions of strict reverse isolation seems unnecessary.

In patients receiving allogeneic bone marrow, the question of what type of antimicrobial prophylaxis to apply, is still open. The Graft versus Host reaction in decontaminated individuals is very much mitigated or even does not occur (Jones, 1971; Van Bekkum, 1974; Heit et al., 1977). A similar observation was made in mice associated with anaerobes only (Van Bekkum, 1977b). Also still open is the question of whether these patients should be submitted to strict reverse isolation when selective decontamination is performed.

SUMMARY

In periods of extreme immunosuppression, infections which are often life-threatening, frequently occur. In an attempt to prevent such infections in lethally irradiated rhesus monkeys, the animals were subjected to strict reverse isolation prior to irradiation and administrated orally with nonabsorbable antibiotics in order to eliminate their microflora. The antibiotic combination was selected on the basis of a sensitivity test and was added to the liquid food supply. To rapidly achieve a high bactericidal concentration in the intestine, the same antibiotics were additionally given orally for 5 days. The microflora was reduced rapidly; within a few days sterile cultures were obtained. Particularly after discontinuation of the administration of the additional antibiotics were colonizations found. In contrast to colonizations persisting from the first day of treatment on, the first were rather easy to suppress.

Due to a lack of typing facilities, all colonizations found after day 7 were considered as exogenous and therefore to represent a break in isolation. As the greatest number of exogenous colonizations were found in the first two weeks of decontamination, it was therefore concluded that these might have been in fact of endogenous origin. It was also concluded that a germfree status was not achieved and an extreme microflora suppression was all that was accomplished.

Besides a decrease of 5% in the haematocrit, no effect of the decontamination was seen on parameters for liver and kidney functions or on electrolyte metabolism or bone marrow function.

After at least two weeks of decontamination, the monkeys were submitted to a total body irradiation of 8.5 Gy, almost always followed by a bone marrow graft. In the following weeks of severe immunodeficiency, a number of colonizations were again found. Some of these were known to be present on the day of irradiation. A number of colonizations were found after irradiation due to microorganisms that colonized the monkey before irradiation, but were suppressed before irradiation. The chance of reappearance of microorganisms proved not to depend on the interval between the latest isolation and irradiation. A number of colonizations were by microorganisms not previously isolated. These were considered as exogenous. It was considered that a number of them still might have been of endogenous origin, since quite a number were found in the first period after irradiation as compared to the later treatment intervals.

A relatively large number of colonizations were of limited dissemination and duration, as indicated by a few isolations only. These disappeared spontaneously, i.e., without adjustment of treatment. An additional number of colonizations required adjustment of therapy on the basis of sensitivity testing for suppression. Only a few colonizations in the irradiated animals could not be controlled; the monkeys died heavily colonized.

Many colonizations started in the oral cavity. The microorganisms isolated were rarely resistant to both antibiotics applied. Although a large number of positive swabs proved to be contaminations, it was thought to be better to perform a sensitivity test on each isolate.

Irradiation caused a severe diarrhoea in decontaminated animals, leading to a life-threatening loss of water and electrolytes. In the first part of the study, addition of potassium chloride to the food was sufficient to compensate for this loss. For not understood reasons this changed during the study, as it suddenly became insufficient. Only when it was realized that the amount of sodium in the food was - and had been - very low and when this was corrected for by additional sodium did the monkeys survive for prolonged times after irradiation.

The antibiotic concentration in the faeces was well above the amount indicated as being required according to the sensitivity test. The amount of antibiotic containing food taken had no effect on the faecal concentration. Addition of solid food particles, however, resulted in a much lower faecal concentration. No significant antibiotic serum levels were found, indicating negligible absorption after irradiation.

To prevent an abnormal colonization of the intestine after discontinuation of the antibiotic treatment, an anaerobic microflora was administered in an attempt to restore the colonization resistance. In the interval between discontinuation of the antibiotics and administration of and intestinal colonization by the donor flora, microorganisms present were "unopposed" and grew out into a high density.

A high bacterial density of potentially pathogenic microorganisms in the oropharyngeal or intestinal flora can easily result in invasion by these microorganisms. This in turn requires the administration of antibiotics. In a mouse model, it was investigated which antibiotics when administered intraperitoneally negatively influenced the donor flora and reduced the colonization resistance. It was shown that erythromycin, clindamycin and carbenicillin suppressed the donor flora permanently, as indicated by a reduced colonization resistance. Benzylpenicillin, ampicillin, doxycycline and the combination gentamicin-

cephalothin affected the colonization resistance as long as these agents were present, while gentamicin alone and cephalothin and oxytetracycline did not affect the colonization resistance.

SAMENVATTING

Gedurende perioden van extreme immunosuppressie zijn infecties frequent en vaak levensbedreigend. Om deze infecties te voorkomen werd voor de immunosuppressieve therapie, onder condities van strikte omgekeerde isolatie, de microflora van rhesus apen met orale, niet-resorbeerbare antibiotica onderdrukt. De op basis van een gevoeligheidstest geselecteerde antibiotica werden aan het - vloeibare - voedsel toegevoegd. Bovendien kregen de apen, om snel een hoge "bactericide" concentratie in de darm te bereiken, gedurende de eerste 5 dagen additionele doses per os. Dit leidde tot een snelle reductie van de microflora. Reeds na enkele dagen bleven de kweken van de faeces monsters steriel. Na het staken van de additionele antibiotica trad een aantal kolonisaties op. Deze waren gemakkelijk te onderdrukken, dit in tegenstelling tot kolonisaties die vanaf het begin van de antibioticum toediening persisteerden.

Microorganismen gevonden na de 7e dag werden geacht tijdens de behandelingsperiode de isolatiebarrière te hebben doorbroken. Het grootste aantal "exogene kolonisaties" trad echter op in de eerste 2 weken van de decontaminatie. Verondersteld werd daarom dat een aantal van deze exogene kolonisaties toch van endogene oorsprong waren. Dit leidde tot de stelling dat een kiemvrije status niet wordt bereikt, maar dat er veel eerder sprake is van een extreme suppressie van de microflora.

Decontaminatie had, behalve een daling van de haematocriet waarde met 5%, geen effect op de lever- en nierfuncties noch op de electrolyten huishouding noch op de beenmerg functies.

Na tenminste 2 weken decontaminatie werden de apen bestraald met een totale lichaamsbestraling van 8,5 Gy, veelal gevolgd door een beenmergtransplantatie. In de hierna volgende weken van sterke immunodeficientie trad ook een aantal kolonisaties op. Een deel van deze kolonisaties was reeds aanwezig tijdens de bestraling. Een aantal van de kolonisaties die voor bestraling werden onderdrukt, keerden na bestraling terug. De kans op dit herverschijnen van microorganismen bleek niet afhankelijk van het tijdsinterval tussen de laatste isolatie en de bestraling. Tenslotte werd een aantal microorganismen geisoleerd, die niet eerder waren gevonden. Aangezien een onevenredig deel van deze kolonisaties in de eerste periode na de bestraling optrad werd ook hier verondersteld dat een gedeelte toch endogeen van oorsprong was.

Hoewel een groot aantal "kolonisaties" werd gevonden bleken de meeste van beperkte omvang en beperkte duur te zijn. Veel kolonisaties, aangegeven door slechts enkele positieve kweken, verdwenen weer zonder aanpassing van de orale antibiotica. Door aanpassing van de orale antibiotica op geleide van het gevoeligheidspatroon van de geisoleerde stammen lukte het een aantal van de overige kolonisaties te onderdrukken. Slechts in enkele gevallen gelukte dit niet; de apen overleden ernstig gekoloniseerd met deze microorganismen.

Veel kolonisaties begonnen in de mondholte. Het betrof dan zelden microorganismen die volledig resistent waren tegen de gebruikte antibiotica. De helft van de positieve kweken bleken "contaminaties" (eenmalige isolaties) te zijn, het moet echter toch raadzaam worden geacht om in alle gevallen een antibiogram te bepalen.

Bestraling leidde altijd tot een ernstige diarree in de gedecontamineerde apen. Dit resulteerde in een levensbedreigend verlies van water en electrolyten. In het eerste deel van de studie werd extra KCl aan de voeding toegevoegd. Dit leek voldoende om het verlies te corrigeren. Om onduidelijke redenen veranderde dit plotseling gedurende de studie en overleefden de apen de bestraling korter dan 2 weken. Pas toen bemerkt werd dat het natriumgehalte van het voedsel (altijd al) extreem laag was en ook dit was gecorrigeerd, overleefden de apen weer gedurende langere tijd na bestraling.

De antibioticum spiegels in de faeces lagen ruim boven de in de gevoeligheidstest bepaalde minimum remmende concentraties. De hoeveelheid (gedronken antibioticum-houdend) voedsel bleek niet van invloed op de antibioticum concentratie in de faeces. Wel waren de spiegels veel lager wanneer ook vast voedsel werd gegeven. In het serum was slechts incidenteel een spoor van de toegediende antibiotica aantoonbaar.

Teneinde te voorkomen dat een abnormaal kolonisatiepatroon van de darm zou ontstaan, werd na het staken van de antibiotica een anaerobe darmflora toegediend om de kolonisatieresistentie te herstellen. Gedurende het interval tussen het verdwijnen van de antibiotica en het toedienen en uitgroeien van de"donor flora"hebben de aanwezige microorganismen "vrij spel". Deze groeiden dan ook uit, koloniseerden de apen en bereikten daarbij hoge concentraties.

Een grote bacteriele populatie dichtheid in de darm of de mondholte kan tot invasie van die microorganismen leiden. Dit vereist weer toediening van antibiotica. In een muize-model werd voor de verschillende klinisch veel gebruikte antibiotica nagegaan welke bij parenterale toediening de "donor flora" onderdrukken en de kolonisatie resistentie verlagen. Clindamycine, erythromycine en carbenicilline

leidden tot een permanente verlaging van de kolonisatie resistentie. Benzylpenicilline, ampicilline, doxycycline en de combinatie gentamicine-cephalothin hadden een negatief effect op de kolonisatie resistentie zolang deze middelen werden toegediend. Geen effect op de kolonisatie resistentie hadden gentamicine, cephalothin en oxytetracycline.

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